

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



12

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/53, 15/82, A01H 5/00	A2	(11) International Publication Number: WO 96/21022 (43) International Publication Date: 11 July 1996 (11.07.96)
(21) International Application Number: PCT/IB95/01167 (22) International Filing Date: 28 December 1995 (28.12.95) (30) Priority Data: 08/366,779 30 December 1994 (30.12.94) US (71) Applicant: RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre-Baizet, F-69263 Lyon (FR). (72) Inventors: THOMAS, Terry, L.; 3004 Normand, College Station, TX 77845 (US). REDDY, Avutu, S.; 3902 E. 29th Street #G11, Bryan, TX 77802 (US). NUCCIO, Michael; P.O. Box 553, College Station, TX 77841 (US). NUNBERG, Andrew, N.; 2804 B. Sprucewood Street, Bryan, TX 77801 (US). FREYSSINET, Georges, L.; 21, rue de Nervieux, F-69450 Saint-Cyr-au-Mont-d'Or (FR). (74) Agent: MITSCHERLICH & PARTNER; Sonnenstrasse 33, D-80331 München (DE).		(81) Designated States: AU, BR, CA, CN, JP, RO, RU, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE (57) Abstract Linoleic acid is converted into γ -linolenic acid by the enzyme $\Delta 6$ -desaturase. The present invention is directed to isolated nucleic acids comprising the $\Delta 6$ -desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the $\Delta 6$ -desaturase gene. The present invention provides recombinant constructions comprising the $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

1 PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE

Linoleic acid (18:2) (LA) is transformed into gamma linolenic acid (18:3) (GLA) by the enzyme
5 $\Delta 6$ -desaturase. When this enzyme, or the nucleic acid encoding it, is transferred into LA-producing cells, GLA is produced. The present invention provides nucleic acids comprising the $\Delta 6$ -desaturase gene. More specifically, the nucleic acids comprise the
10 promoters, coding regions and termination regions of the $\Delta 6$ -desaturase genes. The present invention is further directed to recombinant constructions comprising a $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences.
15 The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

Unsaturated fatty acids such as linoleic ($C_{18}\Delta^{9,12}$) and α -linolenic ($C_{18}\Delta^{9,12,15}$) acids are essential
20 dietary constituents that cannot be synthesized by vertebrates since vertebrate cells can introduce double bonds at the Δ^9 position of fatty acids but cannot introduce additional double bonds between the Δ^9 double bond and the methyl-terminus of the fatty
25 acid chain. Because they are precursors of other products, linoleic and α -linolenic acids are essential fatty acids, and are usually obtained from plant sources. Linoleic acid can be converted by mammals into γ -linolenic acid (GLA, $C_{18}\Delta^{6,9,12}$) which can in turn
30 be converted to arachidonic acid (20:4), a critically

1 important fatty acid since it is an essential precursor of most prostaglandins.

The dietary provision of linoleic acid, by virtue of its resulting conversion to GLA and
5 arachidonic acid, satisfies the dietary need for GLA and arachidonic acid. However, a relationship has been demonstrated between consumption of saturated
10 fats and health risks such as hypercholesterolemia, atherosclerosis and other clinical disorders which correlate with susceptibility to coronary disease, while the consumption of unsaturated fats has been associated with decreased blood cholesterol concentration and reduced risk of atherosclerosis. The therapeutic benefits of dietary GLA may result
15 from GLA being a precursor to arachidonic acid and thus subsequently contributing to prostaglandin synthesis. Accordingly, consumption of the more unsaturated GLA, rather than linoleic acid, has potential health benefits. However, GLA is not
20 present in virtually any commercially grown crop plant.

Linoleic acid is converted into GLA by the enzyme $\Delta 6$ -desaturase. $\Delta 6$ -desaturase, an enzyme of more than 350 amino acids, has a membrane-bound domain
25 and an active site for desaturation of fatty acids. When this enzyme is transferred into cells which endogenously produce linoleic acid but not GLA, GLA is produced. The present invention, by providing the gene encoding $\Delta 6$ -desaturase, allows the production of
30 transgenic organisms which contain functional $\Delta 6$ -desaturase and which produce GLA. In addition to

1 allowing production of large amounts of GLA, the
present invention provides new dietary sources of GLA.

The present invention is directed to
isolated $\Delta 6$ -desaturase genes. Specifically, the
5 isolated genes comprises the $\Delta 6$ -desaturase promoters,
coding regions, and termination regions.

The present invention is further directed to
expression vectors comprising the $\Delta 6$ -desaturase
promoter, coding region and termination region.

10 Yet another aspect of this invention is
directed to expression vectors comprising a $\Delta 6$ -
desaturase coding region in functional combination
with heterologous regulatory regions, i.e. elements
not derived from the $\Delta 6$ -desaturase gene.

15 Cells and organisms comprising the vectors
of the present invention, and progeny of such
organisms, are also provided by the present invention.

A further aspect of the present invention
provides isolated bacterial $\Delta 6$ -desaturase. An
20 isolated plant $\Delta 6$ -desaturase is also provided.

Yet another aspect of this invention
provides a method for producing plants with increased
gamma linolenic acid content.

A method for producing chilling tolerant
25 plants is also provided by the present invention.

Fig. 1 depicts the hydropathy profiles of
the deduced amino acid sequences of Synechocystis $\Delta 6$ -
desaturase (Panel A) and $\Delta 12$ -desaturase (Panel B).
Putative membrane spanning regions are indicated by
30 solid bars. Hydrophobic index was calculated for a

1 window size of 19 amino acid residues [Kyte, et al.
(1982) J. Molec. Biol. 157].

Fig. 2 provides gas liquid chromatography
profiles of wild type (Panel A) and transgenic (Panel
5 B) Anabaena.

Fig. 3 is a diagram of maps of cosmid cSy75,
cSy13 and Csy7 with overlapping regions and subclones.
The origins of subclones of Csy75, Csy75-3.5 and Csy7
are indicated by the dashed diagonal lines.
10 Restriction sites that have been inactivated are in
parentheses.

Fig. 4 provides gas liquid chromatography
profiles of wild type (Panel A) and transgenic (Panel
B) tobacco.

15 Fig. 5A depicts the DNA sequence of a Δ -6
desaturase cDNA isolated from borage.

Fig. 5B depicts the protein sequence of the
open reading frame in the isolated borage Δ -6
desaturase cDNA. Three amino acid motifs
20 characteristic of desaturases are indicated and are,
in order, lipid box, metal box 1, and metal box 2.

Fig. 6 is a dendrogram showing similarity of
the borage Δ 6-desaturase to other membrane-bound
desaturases. The amino acid sequence of the borage
25 Δ 6-desaturase was compared to other known desaturases
using Gene Works (IntelliGenetics). Numerical values
correlate to relative phylogenetic distances between
subgroups compared.

Fig. 7 is a restriction map of 221. Δ 6.NOS
30 and 121. Δ 6.NOS. In 221. Δ 6.NOS, the remaining portion

1 of the plasmid is pBI221 and in 121.Δ6.NOS, the
remaining portion of the plasmid is pBI121.

Fig. 8 provides gas liquid chromatography
profiles of mock transfected (Panel A) and 221.Δ6.NOS
5 transfected (Panel B) carrot cells. The positions of
18:2, 18:3 α, and 18:3 γ(GLA) are indicated.

Fig. 9 provides gas liquid chromatography
profiles of an untransformed tobacco leaf (Panel A)
and a tobacco leaf transformed with 121.Δ6.NOS. The
10 positions of 18:2, 18:3 α, 18:3γ(GLA), and 18:4 are
indicated.

Fig. 10 provides gas liquid chromatography
profiles for untransformed tobacco seeds (Panel A) and
seeds of tobacco transformed with 121.Δ6.NOS. The
15 positions of 18:2, 18:3α and 18:3γ(GLA) are indicated.

The present invention provides isolated
nucleic acids encoding Δ6-desaturase. To identify a
nucleic acid encoding Δ6-desaturase, DNA is isolated
from an organism which produces GLA. Said organism
20 can be, for example, an animal cell, certain fungi
(e.g. Mortierella), certain bacteria (e.g.
Synechocystis) or certain plants (borage, Oenothera,
currants). The isolation of genomic DNA can be
accomplished by a variety of methods well-known to one
25 of ordinary skill in the art, as exemplified by
Sambrook et al. (1989) in Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor, NY. The
isolated DNA is fragmented by physical methods or
enzymatic digestion and cloned into an appropriate
30 vector, e.g. a bacteriophage or cosmid vector, by any
of a variety of well-known methods which can be found

1 in references such as Sambrook et al. (1989).
Expression vectors containing the DNA of the present
invention are specifically contemplated herein. DNA
encoding $\Delta 6$ -desaturase can be identified by gain of
5 function analysis. The vector containing fragmented
DNA is transferred, for example by infection,
transconjugation, transfection, into a host organism
that produces linoleic acid but not GLA. As used
herein, "transformation" refers generally to the
10 incorporation of foreign DNA into a host cell.
Methods for introducing recombinant DNA into a host
organism are known to one of ordinary skill in the art
and can be found, for example, in Sambrook et al.
(1989). Production of GLA by these organisms (i.e.,
15 gain of function) is assayed, for example by gas
chromatography or other methods known to the
ordinarily skilled artisan. Organisms which are
induced to produce GLA, i.e. have gained function by
the introduction of the vector, are identified as
20 expressing DNA encoding $\Delta 6$ -desaturase, and said DNA is
recovered from the organisms. The recovered DNA can
again be fragmented, cloned with expression vectors,
and functionally assessed by the above procedures to
define with more particularity the DNA encoding $\Delta 6$ -
25 desaturase.

As an example of the present invention,
random DNA is isolated from the cyanobacteria
Synechocystis Pasteur Culture Collection (PCC) 6803,
American Type Culture Collection (ATCC) 27184, cloned
30 into a cosmid vector, and introduced by
transconjugation into the GLA-deficient cyanobacterium

1 Anabaena strain PCC 7120, ATCC 27893. Production of GLA from Anabaena linoleic acid is monitored by gas chromatography and the corresponding DNA fragment is isolated.

5 The isolated DNA is sequenced by methods well-known to one of ordinary skill in the art as found, for example, in Sambrook et al. (1989).

In accordance with the present invention, DNA molecules comprising $\Delta 6$ -desaturase genes have been
10 isolated. More particularly, a 3.588 kilobase (kb) DNA comprising a $\Delta 6$ -desaturase gene has been isolated from the cyanobacteria Synechocystis. The nucleotide sequence of the 3.588 kb DNA was determined and is shown in SEQ ID NO:1. Open reading frames defining
15 potential coding regions are present from nucleotide 317 to 1507 and from nucleotide 2002 to 3081. To define the nucleotides responsible for encoding $\Delta 6$ -desaturase, the 3.588 kb fragment that confers $\Delta 6$ -desaturase activity is cleaved into two subfragments,
20 each of which contains only one open reading frame. Fragment ORF1 contains nucleotides 1 through 1704, while fragment ORF2 contains nucleotides 1705 through 3588. Each fragment is subcloned in both forward and reverse orientations into a conjugal expression vector
25 (AM542, Wolk et al. [1984] Proc. Natl. Acad. Sci. USA 81, 1561) that contains a cyanobacterial carboxylase promoter. The resulting constructs (i.e. ORF1(F), ORF1(R), ORF2(F) and ORF2(R)) are conjugated to wild-type Anabaena PCC 7120 by standard methods (see, for
30 example, Wolk et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1561). Conjugated cells of Anabaena are

1 identified as Neo^R green colonies on a brown
background of dying non-conjugated cells after two
weeks of growth on selective media (standard mineral
media BG11N + containing 30µg/ml of neomycin according
5 to Rippka et al., (1979) J. Gen Microbiol. 111, 1).
The green colonies are selected and grown in selective
liquid media (BG11N + with 15µg/ml neomycin). Lipids
are extracted by standard methods (e.g. Dahmer et al.,
(1989) Journal of American Oil Chemical Society 66,
10 543) from the resulting transconjugants containing the
forward and reverse oriented ORF1 and ORF2 constructs.
For comparison, lipids are also extracted from wild-
type cultures of Anabaena and Synechocystis. The
fatty acid methyl esters are analyzed by gas liquid
15 chromatography (GLC), for example with a Tracor-560
gas liquid chromatograph equipped with a hydrogen
flame ionization detector and a capillary column. The
results of GLC analysis are shown in Table 1.

20

25

30

35

1 Table 1: Occurrence of C18 fatty acids in wild-type and transgenic cyanobacteria

	SOURCE	18:0	18:1	18:2	γ 18:3	α 18:3	18:4
5	Anabaena (wild type)	+	+	+	-	+	-
	Anabaena + ORF1(F)	+	+	+	-	+	-
	Anabaena + ORF1(R)	+	+	+	-	+	-
	Anabaena + ORF2(F)	+	+	+	+	+	+
10	Anabaena + ORF2(R)	+	+	+	-	+	-
	Synechocystis (wild type)	+	+	+	+	-	-

As assessed by GLC analysis, GLA deficient Anabaena gain the function of GLA production when the construct containing ORF2 in forward orientation is introduced by transconjugation. Transconjugants containing constructs with ORF2 in reverse orientation to the carboxylase promoter, or ORF1 in either orientation, show no GLA production. This analysis demonstrates that the single open reading frame (ORF2) within the 1884 bp fragment encodes Δ 6-desaturase. The 1884 bp fragment is shown as SEQ ID NO:3. This is substantiated by the overall similarity of the hydropathy profiles between Δ 6-desaturase and Δ 12-desaturase [Wada et al. (1990) Nature 347] as shown in Fig. 1 as (A) and (B), respectively.

Also in accordance with the present invention, a cDNA comprising a Δ 6-desaturase gene from borage (Borago officinalis) has been isolated. The nucleotide sequence of the 1.685 kilobase (kb) cDNA

1 was determined and is shown in Fig. 5A (SEQ ID NO: 4).
The ATG start codon and stop codon are underlined.
The amino acid sequence corresponding to the open
reading frame in the borage delta 6-desaturase is
5 shown in Fig. 5B (SEQ ID NO: 5).

Isolated nucleic acids encoding $\Delta 6$ -
desaturase can be identified from other GLA-producing
organisms by the gain of function analysis described
above, or by nucleic acid hybridization techniques
10 using the isolated nucleic acid which encodes
Synechocystis or borage $\Delta 6$ -desaturase as a
hybridization probe. Both genomic and cDNA cloning
methods are known to the skilled artisan and are
contemplated by the present invention. The
15 hybridization probe can comprise the entire DNA
sequence disclosed as SEQ. ID NO:1 or SEQ. ID NO:4, or
a restriction fragment or other DNA fragment thereof,
including an oligonucleotide probe. Methods for
cloning homologous genes by cross-hybridization are
20 known to the ordinarily skilled artisan and can be
found, for example, in Sambrook (1989) and Beltz et
al. (1983) Methods in Enzymology 100, 266.

In another method of identifying a delta 6-
desaturase gene from an organism producing GLA, a cDNA
25 library is made from poly-A⁺ RNA isolated from
polysomal RNA. In order to eliminate hyper-abundant
expressed genes from the cDNA population, cDNAs or
fragments thereof corresponding to hyper-abundant
cDNAs genes are used as hybridization probes to the
30 cDNA library. Non hybridizing plaques are excised and
the resulting bacterial colonies are used to inoculate

1 liquid cultures and sequenced. For example, as a
means of eliminating other seed storage protein cDNAs
from a cDNA library made from borage polysomal RNA,
cDNAs corresponding to abundantly expressed seed
5 storage proteins are first hybridized to the cDNA
library. The "subtracted" DNA library is then used to
generate expressed sequence tags (ESTs) and such tags
are used to scan a data base such as GenBank to
identify potential desaturates.

10 Transgenic organisms which gain the function
of GLA production by introduction of DNA encoding Δ -
desaturase also gain the function of
octadecatetraenoic acid ($18:4^{\Delta 6,9,12,15}$) production.
Octadecatetraenoic acid is present normally in fish
15 oils and in some plant species of the Boraginaceae
family (Craig et al. [1964] J. Amer. Oil Chem. Soc.
41, 209-211; Gross et al. [1976] Can. J. Plant Sci.
56, 659-664). In the transgenic organisms of the
present invention, octadecatetraenoic acid results
20 from further desaturation of α -linolenic acid by $\Delta 6$ -
desaturase or desaturation of GLA by $\Delta 15$ -desaturase.

The 359 amino acids encoded by ORF2, i.e.
the open reading frame encoding Synechocystis $\Delta 6$ -
desaturase, are shown as SEQ. ID NO:2. The open
25 reading frame encoding the borage $\Delta 6$ -desaturase is
shown in SEQ ID NO: 5. The present invention further
contemplates other nucleotide sequences which encode
the amino acids of SEQ ID NO:2 and SEQ ID NO: 5. It
is within the ken of the ordinarily skilled artisan to
30 identify such sequences which result, for example,
from the degeneracy of the genetic code. Furthermore,

1 one of ordinary skill in the art can determine, by the
gain of function analysis described hereinabove,
smaller subfragments of the fragments containing the
open reading frames which encode $\Delta 6$ -desaturases.

5 The present invention contemplates any such
polypeptide fragment of $\Delta 6$ -desaturase and the nucleic
acids therefor which retain activity for converting LA
to GLA.

10 In another aspect of the present invention,
a vector containing a nucleic acid of the present
invention or a smaller fragment containing the
promoter, coding sequence and termination region of a
 $\Delta 6$ -desaturase gene is transferred into an organism,
for example, cyanobacteria, in which the $\Delta 6$ -desaturase
15 promoter and termination regions are functional.
Accordingly, organisms producing recombinant $\Delta 6$ -
desaturase are provided by this invention. Yet
another aspect of this invention provides isolated $\Delta 6$ -
desaturase, which can be purified from the recombinant
20 organisms by standard methods of protein purification.
(For example, see Ausubel et al. [1987] Current
Protocols in Molecular Biology, Green Publishing
Associates, New York).

25 Vectors containing DNA encoding $\Delta 6$ -
desaturase are also provided by the present invention.
It will be apparent to one of ordinary skill in the
art that appropriate vectors can be constructed to
direct the expression of the $\Delta 6$ -desaturase coding
sequence in a variety of organisms. Replicable
expression vectors are particularly preferred.
30 Replicable expression vectors as described herein are

1 DNA or RNA molecules engineered for controlled
expression of a desired gene, i.e. the $\Delta 6$ -desaturase
gene. Preferably the vectors are plasmids,
bacteriophages, cosmids or viruses. Shuttle vectors,
5 e.g. as described by Wolk et al. (1984) Proc. Natl.
Acad. Sci. USA, 1561-1565 and Bustos et al. (1991) J.
Bacteriol. 174, 7525-7533, are also contemplated in
accordance with the present invention. Sambrook et
al. (1989), Goeddel, ed. (1990) Methods in Enzymology
10 185 Academic Press, and Perbal (1988) A Practical
Guide to Molecular Cloning, John Wiley and Sons, Inc.,
provide detailed reviews of vectors into which a
nucleic acid encoding the present $\Delta 6$ -desaturase can be
inserted and expressed. Such vectors also contain
15 nucleic acid sequences which can effect expression of
nucleic acids encoding $\Delta 6$ -desaturase. Sequence
elements capable of effecting expression of a gene
product include promoters, enhancer elements, upstream
activating sequences, transcription termination
20 signals and polyadenylation sites. Both constitutive
and tissue specific promoters are contemplated. For
transformation of plant cells, the cauliflower mosaic
virus (CaMV) 35S promoter and promoters which are
regulated during plant seed maturation are of
25 particular interest. All such promoter and
transcriptional regulatory elements, singly or in
combination, are contemplated for use in the present
replicable expression vectors and are known to one of
ordinary skill in the art. The CaMV 35S promoter is
30 described, for example, by Restrepo et al. (1990)

1 Plant Cell 2, 987. Genetically engineered and mutated
regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine
vectors and regulatory elements suitable for
5 expression in a particular host cell. For example, a
vector comprising the promoter from the gene encoding
the carboxylase of Anabaena operably linked to the
coding region of $\Delta 6$ -desaturase and further operably
linked to a termination signal from Synechocystis is
10 appropriate for expression of $\Delta 6$ -desaturase in
cyanobacteria. "Operably linked" in this context
means that the promoter and terminator sequences
effectively function to regulate transcription. As a
further example, a vector appropriate for expression
15 of $\Delta 6$ -desaturase in transgenic plants can comprise a
seed-specific promoter sequence derived from
helianthinin, napin, or glycinin operably linked to
the $\Delta 6$ -desaturase coding region and further operably
linked to a seed termination signal or the nopaline
20 synthase termination signal. As a still further
example, a vector for use in expression of $\Delta 6$ -
desaturase in plants can comprise a constitutive
promoter or a tissue specific promoter operably linked
to the $\Delta 6$ -desaturase coding region and further
25 operably linked to a constitutive or tissue specific
terminator or the nopaline synthase termination
signal.

In particular, the helianthinin regulatory
elements disclosed in applicant's copending U.S.
30 Application Serial No. 682,354, filed April 8, 1991
and incorporated herein by reference, are contemplated

1 as promoter elements to direct the expression of the
Δ6-desaturase of the present invention.

Modifications of the nucleotide sequences or
regulatory elements disclosed herein which maintain
5 the functions contemplated herein are within the scope
of this invention. Such modifications include
insertions, substitutions and deletions, and
specifically substitutions which reflect the
degeneracy of the genetic code.

10 Standard techniques for the construction of
such hybrid vectors are well-known to those of
ordinary skill in the art and can be found in
references such as Sambrook et al. (1989), or any of
the myriad of laboratory manuals on recombinant DNA
15 technology that are widely available. A variety of
strategies are available for ligating fragments of
DNA, the choice of which depends on the nature of the
termini of the DNA fragments. It is further
contemplated in accordance with the present invention
20 to include in the hybrid vectors other nucleotide
sequence elements which facilitate cloning, expression
or processing, for example sequences encoding signal
peptides, a sequence encoding KDEL, which is required
for retention of proteins in the endoplasmic reticulum
25 or sequences encoding transit peptides which direct
Δ6-desaturase to the chloroplast. Such sequences are
known to one of ordinary skill in the art. An
optimized transit peptide is described, for example,
by Van den Broeck et al. (1985) Nature 313, 358.
30 Prokaryotic and eukaryotic signal sequences are

35

1 disclosed, for example, by Michaelis et al. (1982)
Ann. Rev. Microbiol. 36, 425.

5 A further aspect of the instant invention
provides organisms other than cyanobacteria or plants
which contain the DNA encoding the $\Delta 6$ -desaturase of
the present invention. The transgenic organisms
contemplated in accordance with the present invention
include bacteria, cyanobacteria, fungi, and plants and
animals. The isolated DNA of the present invention
10 can be introduced into the host by methods known in
the art, for example infection, transfection,
transformation or transconjugation. Techniques for
transferring the DNA of the present invention into
such organisms are widely known and provided in
15 references such as Sambrook et al. (1989).

A variety of plant transformation methods
are known. The $\Delta 6$ -desaturase gene can be introduced
into plants by a leaf disk transformation-regeneration
procedure as described by Horsch et al. (1985) Science
20 227, 1229. Other methods of transformation, such as
protoplast culture (Horsch et al. (1984) Science 223,
496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et
al. (1983) Cell 32, 1033) can also be used and are
within the scope of this invention. In a preferred
25 embodiment plants are transformed with Agrobacterium-
derived vectors. However, other methods are available
to insert the $\Delta 6$ -desaturase genes of the present
invention into plant cells. Such alternative methods
include biolistic approaches (Klein et al. (1987)
30 Nature 327, 70), electroporation, chemically-induced
DNA uptake, and use of viruses or pollen as vectors.

1 When necessary for the transformation
method, the $\Delta 6$ -desaturase genes of the present
invention can be inserted into a plant transformation
vector, e.g. the binary vector described by Bevan
5 (1984) Nucleic Acids Res. 12, 8111. Plant
transformation vectors can be derived by modifying the
natural gene transfer system of Agrobacterium
tumefaciens. The natural system comprises large Ti
(tumor-inducing)-plasmids containing a large segment,
10 known as T-DNA, which is transferred to transformed
plants. Another segment of the Ti plasmid, the vir
region, is responsible for T-DNA transfer. The T-DNA
region is bordered by terminal repeats. In the
modified binary vectors the tumor-inducing genes have
15 been deleted and the functions of the vir region are
utilized to transfer foreign DNA bordered by the T-DNA
border sequences. The T-region also contains a
selectable marker for antibiotic resistance, and a
multiple cloning site for inserting sequences for
20 transfer. Such engineered strains are known as
"disarmed" A. tumefaciens strains, and allow the
efficient transformation of sequences bordered by the
T-region into the nuclear genomes of plants.

Surface-sterilized leaf disks are inoculated
25 with the "disarmed" foreign DNA-containing A.
tumefaciens, cultured for two days, and then
transferred to antibiotic-containing medium.
Transformed shoots are selected after rooting in
medium containing the appropriate antibiotic,
30 transferred to soil and regenerated.

1 Another aspect of the present invention
provides transgenic plants or progeny of these plants
containing the isolated DNA of the invention. Both
monocotyledenous and dicotyledenous plants are
5 contemplated. Plant cells are transformed with the
isolated DNA encoding $\Delta 6$ -desaturase by any of the
plant transformation methods described above. The
transformed plant cell, usually in a callus culture or
leaf disk, is regenerated into a complete transgenic
10 plant by methods well-known to one of ordinary skill
in the art (e.g. Horsch et al. (1985) Science 227,
1129). In a preferred embodiment, the transgenic
plant is sunflower, oil seed rape, maize, tobacco,
peanut or soybean. Since progeny of transformed
15 plants inherit the DNA encoding $\Delta 6$ -desaturase, seeds
or cuttings from transformed plants are used to
maintain the transgenic plant line.

The present invention further provides a
method for providing transgenic plants with an
20 increased content of GLA. This method includes
introducing DNA encoding $\Delta 6$ -desaturase into plant
cells which lack or have low levels of GLA but contain
LA, and regenerating plants with increased GLA content
from the transgenic cells. In particular,
25 commercially grown crop plants are contemplated as the
transgenic organism, including, but not limited to,
sunflower, soybean, oil seed rape, maize, peanut and
tobacco.

The present invention further provides a
30 method for providing transgenic organisms which
contain GLA. This method comprises introducing DNA

1 encoding $\Delta 6$ -desaturase into an organism which lacks or
has low levels of GLA, but contains LA. In another
embodiment, the method comprises introducing one or
more expression vectors which comprise DNA encoding
5 $\Delta 12$ -desaturase and $\Delta 6$ -desaturase into organisms which
are deficient in both GLA and LA. Accordingly,
organisms deficient in both LA and GLA are induced to
produce LA by the expression of $\Delta 12$ -desaturase, and
GLA is then generated due to the expression of $\Delta 6$ -
10 desaturase. Expression vectors comprising DNA
encoding $\Delta 12$ -desaturase, or $\Delta 12$ -desaturase and $\Delta 6$ -
desaturase, can be constructed by methods of
recombinant technology known to one of ordinary skill
in the art (Sambrook et al., 1989) and the published
15 sequence of $\Delta 12$ -desaturase (Wada et al. [1990] Nature
(London) 347, 200-203. In addition, it has been
discovered in accordance with the present invention
that nucleotides 2002-3081 of SEQ. ID NO:1 encode
cyanobacterial $\Delta 12$ -desaturase. Accordingly, this
20 sequence can be used to construct the subject
expression vectors. In particular, commercially grown
crop plants are contemplated as the transgenic
organism, including, but not limited to, sunflower,
soybean, oil seed rape, maize, peanut and tobacco.
25 The present invention is further directed to
a method of inducing chilling tolerance in plants.
Chilling sensitivity may be due to phase transition of
lipids in cell membranes. Phase transition
temperature depends upon the degree of unsaturation of
fatty acids in membrane lipids, and thus increasing
30 the degree of unsaturation, for example by introducing

1 Δ 6-desaturase to convert LA to GLA, can induce or
improve chilling resistance. Accordingly, the present
method comprises introducing DNA encoding Δ 6-
desaturase into a plant cell, and regenerating a plant
5 with improved chilling resistance from said
transformed plant cell. In a preferred embodiment,
the plant is a sunflower, soybean, oil seed rape,
maize, peanut or tobacco plant.

The following examples further illustrate
10 the present invention.

15

20

25

30

35

1

EXAMPLE 1

Strains and Culture Conditions

- Synechocystis (PCC 6803, ATCC 27184),
5 Anabaena (PCC 7120, ATCC 27893) and Synechococcus (PCC
7942, ATCC 33912) were grown photoautotrophically at
30°C in BG11N+ medium (Rippka et al. [1979] J. Gen.
Microbiol. 111, 1-61) under illumination of
incandescent lamps
10 ($60\mu\text{E}\cdot\text{m}^{-2}\cdot\text{S}^{-1}$). Cosmids and plasmids were selected and
propagated in Escherichia coli strain DH5 α on LB
medium supplemented with antibiotics at standard
concentrations as described by Maniatis et al. (1982)
Molecular Cloning: A Laboratory Manual, Cold Spring
15 Harbor Laboratory, Cold Spring, New York.

20

25

30

35

EXAMPLE 2

1 Construction of Synechocystis Cosmid Genomic Library

5 Total genomic DNA from Synechocystis (PCC
6803) was partially digested with Sau3A and
fractionated on a sucrose gradient (Ausubel et al.
[1987] Current Protocols in Molecular Biology, Greene
Publishing Associates and Wiley Interscience, New
York). Fractions containing 30 to 40 kb DNA fragments
10 were selected and ligated into the dephosphorylated
BamHI site of the cosmid vector, pDUCA7 (Buikema et
al. [1991] J. Bacteriol. 173, 1879-1885). The ligated
DNA was packaged in vitro as described by Ausubel et
al. (1987), and packaged phage were propagated in E.
15 coli DH5 α containing the AvaI and Eco4711 methylase
helper plasmid, pRL528 as described by Buikema et al.
(1991). A total of 1152 colonies were isolated
randomly and maintained individually in twelve 96-well
microtiter plates.

20

25

30

35

EXAMPLE 3

Gain-of-Function Expression of GLA in Anabaena

Anabaena (PCC 7120), a filamentous cyanobacterium, is deficient in GLA but contains significant amounts of linoleic acid, the precursor for GLA (Figure 2; Table 2). The Synechocystis cosmid library described in Example 2 was conjugated into Anabaena (PCC 7120) to identify transconjugants that produce GLA. Anabaena cells were grown to mid-log phase in BG11N+ liquid medium and resuspended in the same medium to a final concentration of approximately 2×10^8 cells per ml. A mid-log phase culture of E. coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol. 114, 341-348) grown in LB containing ampicillin was washed and resuspended in fresh LB medium. Anabaena and RP4 were then mixed and spread evenly on BG11N+ plates containing 5% LB. The cosmid genomic library was replica plated onto LB plates containing 50 μ g/ml kanamycin and 17.5 μ g/ml chloramphenicol and was subsequently patched onto BG11N+ plates containing Anabaena and RP4. After 24 hours of incubation at 30°C, 30 μ g/ml of neomycin was underlaid; and incubation at 30°C was continued until transconjugants appeared.

Individual transconjugants were isolated after conjugation and grown in 2 ml BG11N+ liquid medium with 15 μ g/ml neomycin. Fatty acid methyl esters were prepared from wild type cultures and cultures containing pools of ten transconjugants as follows. Wild type and transgenic cyanobacterial

1 cultures were harvested by centrifugation and washed
twice with distilled water. Fatty acid methyl esters
were extracted from these cultures as described by
Dahmer et al. (1989) J. Amer. Oil. Chem. Soc. 66, 543-
5 548 and were analyzed by Gas Liquid Chromatography
(GLC) using a Tracor-560 equipped with a hydrogen
flame ionization detector and capillary column (30 m x
0.25 mm bonded FSOT Superox II, Alltech Associates
Inc., IL). Retention times and co-chromatography of
10 standards (obtained from Sigma Chemical Co.) were used
for identification of fatty acids. The average fatty
acid composition was determined as the ratio of peak
area of each C18 fatty acid normalized to an internal
standard.

15 Representative GLC profiles are shown in
Fig. 2. C18 fatty acid methyl esters are shown.
Peaks were identified by comparing the elution times
with known standards of fatty acid methyl esters and
were confirmed by gas chromatography-mass
20 spectrometry. Panel A depicts GLC analysis of fatty
acids of wild type Anabaena. The arrow indicates the
migration time of GLA. Panel B is a GLC profile of
fatty acids of transconjugants of Anabaena with
pAM542+1.8F. Two GLA producing pools (of 25 pools
25 representing 250 transconjugants) were identified that
produced GLA. Individual transconjugants of each GLA
positive pool were analyzed for GLA production; two
independent transconjugants, AS13 and AS75, one from
each pool, were identified which expressed significant
30 levels of GLA and which contained cosmids, cSy13 and
cSy75, respectively (Figure 3). The cosmids overlap

1 in a region approximately 7.5 kb in length. A 3.5 kb
NheI fragment of cSy75 was recloned in the vector
pDUCA7 and transferred to Anabaena resulting in gain-
of-function expression of GLA (Table 2).

5 Two NheI/Hind III subfragments (1.8 and 1.7
kb) of the 3.5 kb Nhe I fragment of cSy75-3.5 were
subcloned into "pBLUESCRIPT" (Stratagene) (Figure 3)
for sequencing. Standard molecular biology techniques
were performed as described by Maniatis et al. (1982)
10 and Ausubel et al. (1987). Dideoxy sequencing (Sanger
et al. [1977] Proc. Natl. Acad. Sci. USA 74, 5463-
5467) of pBS1.8 was performed with "SEQUENASE" (United
States Biochemical) on both strands by using specific
oligonucleotide primers synthesized by the Advanced
15 DNA Technologies Laboratory (Biology Department, Texas
A & M University). DNA sequence analysis was done
with the GCG (Madison, WI) software as described by
Devereux et al. (1984) Nucleic Acids Res. 12, 387-395.

Both NheI/HindIII subfragments were
20 transferred into a conjugal expression vector, AM542,
in both forward and reverse orientations with respect
to a cyanobacterial carboxylase promoter and were
introduced into Anabaena by conjugation.
Transconjugants containing the 1.8 kb fragment in the
25 forward orientation (AM542-1.8F) produced significant
quantities of GLA and octadecatetraenoic acid (Figure
2; Table 2). Transconjugants containing other
constructs, either reverse oriented 1.8 kb fragment or
forward and reverse oriented 1.7 kb fragment, did not
30 produce detectable levels of GLA (Table 2).

1 Figure 2 compares the C18 fatty acid profile
of an extract from wild type Anabaena (Figure 2A) with
that of transgenic Anabaena containing the 1.8 kb
fragment of cSy75-3.5 in the forward orientation
5 (Figure 2B). GLC analysis of fatty acid methyl esters
from AM542-1.8F revealed a peak with a retention time
identical to that of authentic GLA standard. Analysis
of this peak by gas chromatography-mass spectrometry
(GC-MS) confirmed that it had the same mass
10 fragmentation pattern as a GLA reference sample.
Transgenic Anabaena with altered levels of
polyunsaturated fatty acids were similar to wild type
in growth rate and morphology.

15

20

25

30

35

1 Table 2 Composition of C18 Fatty Acids in Wild Type
and Transgenic Cyanobacteria

5	Strain	Fatty Acid (%)					
		18:0	18:1	18:2	18:3(α)	18:3(γ)	18:4
10	Wild Type						
	<i>Synechocystis</i>	13.6	4.5	54.5	-	27.3	-
	(sp. PCC6803)						
15	<i>Anabaena</i>	2.9	24.8	37.1	35.2	-	-
	(sp. PCC7120)						
	<i>Synechococcus</i>	20.6	79.4	-	-	-	-
	(sp. PCC7942)						
Anabaena Transconjugants							
20	cSy75	3.8	24.4	22.3	9.1	27.9	12.5
	cSy75-3.5	4.3	27.6	18.1	3.2	40.4	6.4
	pAM542 - 1.8F	4.2	13.9	12.1	19.1	25.4	25.4
	pAM542 - 1.8R	7.7	23.1	38.4	30.8	-	-
	pAM542 - 1.7F	2.8	27.8	36.1	33.3	-	-
	pAM542 - 1.7R	2.8	25.4	42.3	29.6	-	-
Synechococcus Transformants							
25	pAM854	27.8	72.2	-	-	-	-
	pAM854 - Δ^{12}	4.0	43.2	46.0	-	-	-
	pAM854 - Δ^6	18.2	81.8	-	-	-	-
	pAM854 - $\Delta^6\&\Delta^{12}$	42.7	25.3	19.5	-	16.5	-
30	18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid;						
	18:3(α), linolenic acid; 18:3(γ), γ -linolenic acid; 18:4, octadecatetraenoic acid						

EXAMPLE 4

Transformation of Synechococcus
with $\Delta 6$ and $\Delta 12$ Desaturase Genes

1
5 A third cosmid, cSy7, which contains a $\Delta 12$ -
desaturase gene, was isolated by screening the
Synechocystis genomic library with a oligonucleotide
synthesized from the published Synechocystis $\Delta 12$ -
desaturase gene sequence (Wada et al. [1990] Nature
10 (London) 347, 200-203). A 1.7 kb AvaI fragment from
this cosmid containing the $\Delta 12$ -desaturase gene was
identified and used as a probe to demonstrate that
cSy13 not only contains a $\Delta 6$ -desaturase gene but also
a $\Delta 12$ -desaturase gene (Figure 3). Genomic Southern
15 blot analysis further showed that both the $\Delta 6$ -and $\Delta 12$ -
desaturase genes are unique in the Synechocystis
genome so that both functional genes involved in C18
fatty acid desaturation are linked closely in the
Synechocystis genome.

20 The unicellular cyanobacterium Synechococcus
(PCC 7942) is deficient in both linoleic acid and
GLA(3). The $\Delta 12$ and $\Delta 6$ -desaturase genes were cloned
individually and together into pAM854 (Bustos et al.
[1991] J. Bacteriol. 174, 7525-7533), a shuttle vector
that contains sequences necessary for the integration
25 of foreign DNA into the genome of Synechococcus
(Golden et al. [1987] Methods in Enzymol. 153, 215-
231). Synechococcus was transformed with these gene
constructs and colonies were selected. Fatty acid
methyl esters were extracted from transgenic
30 Synechococcus and analyzed by GLC.

1 Table 2 shows that the principal fatty acids
of wild type Synechococcus are stearic acid (18:0) and
oleic acid (18:1). Synechococcus transformed with
pAM854- Δ 12 expressed linoleic acid (18:2) in addition
5 to the principal fatty acids. Transformants with
pAM854- Δ 6 and Δ 12 produced both linoleate and GLA
(Table 1). These results indicated that Synechococcus
containing both Δ 12- and Δ 6-desaturase genes has
gained the capability of introducing a second double
10 bond at the Δ 12 position and a third double bond at
the Δ 6 position of C18 fatty acids. However, no
changes in fatty acid composition was observed in the
transformant containing pAM854- Δ 6, indicating that in
the absence of substrate synthesized by the Δ 12
15 desaturase, the Δ 6-desaturase is inactive. This
experiment further confirms that the 1.8 kb
NheI/HindIII fragment (Figure 3) contains both coding
and promoter regions of the Synechocystis Δ 6-
desaturase gene. Transgenic Synechococcus with
20 altered levels of polyunsaturated fatty acids were
similar to wild type in growth rate and morphology.

25

30

35

EXAMPLE 5

Nucleotide Sequence of $\Delta 6$ -Desaturase

1
5 The nucleotide sequence of the 1.8 kb
fragment of cSy75-3.5 including the functional $\Delta 6$ -
desaturase gene was determined. An open reading frame
encoding a polypeptide of 359 amino acids was
identified (Figure 4). A Kyte-Doolittle hydropathy
analysis (Kyte et al. [1982] J. Mol. Biol. 157, 105-
10 132) identified two regions of hydrophobic amino acids
that could represent transmembrane domains (Figure
1A); furthermore, the hydropathic profile of the $\Delta 6$ -
desaturase is similar to that of the $\Delta 12$ -desaturase
gene (Figure 1B; Wada et al.) and $\Delta 9$ -desaturases
15 (Thiede et al. [1986] J. Biol. Chem. 261, 13230-
13235). However, the sequence similarity between the
Synechocystis $\Delta 6$ - and $\Delta 12$ -desaturases is less than 40%
at the nucleotide level and approximately 18% at the
amino acid level.

20

25

30

35

1

EXAMPLE 6

Transfer of Cyanobacterial Δ^6 -Desaturase into Tobacco

The cyanobacterial Δ^6 -desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis Δ -desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter derived from the sunflower helianthinin gene to drive Δ^6 -desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extensin gene or sunflower helianthinin gene to target newly synthesized Δ^6 -desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the Δ^6 -desaturase ORF, and (iv) an optimized transit peptide to target Δ^6 desaturase into the chloroplast. The 35S promoter is a derivative of pRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al. (1985) EMBO J. 9, 2145.

Transgenic tobacco plants were produced containing a chimeric cyanobacterial desaturase gene,

35

1 comprised of the Synechocystis Δ^6 desaturase gene fused
to an endoplasmic reticulum retention sequence (KDEL)
and extensin signal peptide driven by the CaMV 35S
promoter. PCR amplifications of transgenic tobacco
5 genomic DNA indicate that the Δ^6 desaturase gene was
incorporated into the tobacco genome. Fatty acid
methyl esters of leaves of these transgenic tobacco
plants were extracted and analyzed by Gas Liquid
Chromatography (GLC). These transgenic tobacco
10 accumulated significant amounts of GLA (Figure 4).
Figure 4 shows fatty acid methyl esters as determined
by GLC. Peaks were identified by comparing the
elution times with known standards of fatty acid
methyl ester. Accordingly, cyanobacterial genes
15 involved in fatty acid metabolism can be used to
generate transgenic plants with altered fatty acid
compositions.

20

25

30

35

1

EXAMPLE 7

Construction of Borage cDNA library

Membrane bound polysomes were isolated from
5 borage seeds 12 days post pollination (12 DPP) using
the protocol established for peas by Larkins and
Davies (1975 Plant Phys. 55:749-756). RNA was
extracted from the polysomes as described by Mechler
(1987 Methods in Enzymology 152:241-248, Academic
10 Press).

Poly-A+ RNA was isolated from the membrane
bound polysomal RNA by use of Oligotex-dT beads
(Qiagen). Corresponding cDNA was made using
Stratagene's ZAP cDNA synthesis kit. The cDNA library
15 was constructed in the lambda ZAP II vector
(Stratagene) using the lambda ZAP II vector kit. The
primary library was packaged in Gigapack II Gold
packaging extract (Stratagene). The library was used
to generate expressed sequence tags (ESTs), and
20 sequences corresponding to the tags were used to scan
the GenBank database.

25

30

35

1

EXAMPLE 8
Hybridization Protocol

Hybridization probes for screening the
5 storage cDNA library were generated by using random
primed DNA synthesis as described by Ausubel et al
(1994 Current Protocols in Molecular Biology, Wiley
Interscience, N.Y.) and corresponded to previously
10 identified abundantly expressed seed storage protein
cDNAs. Unincorporated nucleotides were removed by use
of a G-50 spin column (Boehringer Mannheim). Probe was
denatured for hybridization by boiling in a water bath
for 5 minutes, then quickly cooled on ice. Filters
15 for hybridization were prehybridized at 60°C for 2-4
hours in prehybridization solution (6XSSC [Maniatis et
al 1984 Molecular Cloning A Laboratory Manual, Cold
Spring Harbor Laboratory], 1X Denharts Solution, 0.05%
sodium pyrophosphate, 100 µg/ml denatured salmon sperm
20 DNA). Denatured probe was added to the hybridization
solution (6X SSC, 1X Denharts solution, 0.05% sodium
pyrophosphate, 100 µg/ml denatured salmon sperm DNA)
and incubated at 60°C with agitation overnight.
Filters were washed in 4x, 2x, and 1x SET washes for
15 minutes each at 60°C. A 20X SET stock solution is
25 3M NaCl, 0.4 M Tris base, 20 mM Na₂EDTA-2H₂O. The 4X
SET wash was 4X SET, 12.5 mM PO₄, pH 6.8 and 0.2% SDS.
The 2X SET wash was 2X SET, 12.5 mM PO₄, pH 6.8 and
0.2% SDS. The 1X SET wash was 1X SET, 12.5 mM PO₄, pH
6.8 and 0.2% SDS. Filters were allowed to air dry and
30 were then exposed to X-ray film for 24 hours with
intensifying screens at -80°C.

35

1

EXAMPLE 9

Random sequencing of cDNAs from a borage seed
(12 DPP) membrane-bound polysomal library

5

The borage cDNA library was plated at low density (500 pfu on 150 mm petri dishes). Highly prevalent seed storage protein cDNAs were "subtracted" by screening with the previously identified corresponding cDNAs. Non-hybridizing plaques were excised using Stratagene's excision protocol and reagents. Resulting bacterial colonies were used to inoculate liquid cultures and were either sequenced manually or by an ABI automated sequencer. Each cDNA was sequenced once and a sequence tag generated from 200-300 base pairs. All sequencing was performed by cycle sequencing (Epicentre). Over 300 ESTs were generated. Each sequence tag was compared to GenBank database by BLASTX computer program and a number of lipid metabolism genes, including the $\Delta 6$ -desaturase were identified.

20

Database searches with a cDNA clone designated mbp-65 using BLASTX with the GenBank database resulted in a significant match to the Synechocystis $\Delta 6$ -desaturase. It was determined however, that this clone was not a full length cDNA. A full length cDNA was isolated using mbp-65 to screen the borage membrane-bound polysomal library. The sequence of the isolated cDNA was determined (Fig. 5A, SEQ ID NO:4) and the protein sequence of the open reading frame (Fig. 5B, SEQ ID NO:5) was compared to other known desaturases using Geneworks

25

30

35

1 (IntelligGenetics) protein alignment program (Fig. 2).
This alignment indicated that the cDNA was the borage
Δ6-desaturase gene.

5 Although similar to other known plant
desaturases, the borage delta 6-desaturase is distinct
as indicated in the dendrogram shown in Fig. 6.
Furthermore, comparison of the amino acid sequences
characteristic of desaturases, particularly those
proposed to be involved in metal binding (metal box 1
10 and metal box 2), illustrates the differences between
the borage delta 6-desaturase and other plant
desaturases (Table 3).

The borage delta 6-desaturase is
distinguished from the cyanobacterial form not only in
15 over all sequence (Fig. 6) but also in the lipid box,
metal box 1 and metal box 2 amino acid motifs (Table
3). As Table 3 indicates, all three motifs are novel
in sequence. Only the borage delta 6-desaturase metal
box 2 shown some relationship to the Synechocystis
20 delta-6 desaturase metal box 2.

In addition, the borage delta 6-desaturase
is also distinct from another borage desaturase gene,
the delta-12 desaturase. P1-81 is a full length cDNA
that was identified by EST analysis and shows high
25 similarity to the Arabidopsis delta-12 desaturase (Fad
2). A comparison of the lipid box, metal box 1 and
metal box 2 amino acid motifs (Table 3) in borage
delta 6 and delta-12 desaturases indicates that little
homology exists in these regions. The placement of
30 the two sequences in the dendrogram in Fig. 6
indicates how distantly related these two genes are.

Table 3. Comparison of common amino acid motifs in membrane-bound desaturases

Desaturase	Amino Acid Motif					
	Lipid Box	Metal Box 1		Metal Box 2		
Borage Δ^6	WIGHDAGH (SEQ. ID. NO: 6)	HNAHH (SEQ. ID. NO: 12)	FQIEHH (SEQ. ID. NO: 20)			
Synechocystis Δ^6	NVGH DANH (SEQ. ID. NO: 7)	HNYLHH (SEQ. ID. NO: 13)	HQVTHH (SEQ. ID. NO: 21)			
Arab. chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Rice Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Glycine chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Arab. fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Brassica fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Borage Δ^{12} (Pl-81)*	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)			
Arab. fad2 (Δ^{17})	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)			
Arab. chloroplast Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)			
Glycine plastid Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)			
Spinach plastidial n-6	VIGHDCAH (SEQ. ID. NO: 10)	HDQHH (SEQ. ID. NO: 17)	HIPHH (SEQ. ID. NO: 24)			
Synechocystis Δ^{12}	VVGHDCGH (SEQ. ID. NO: 11)	HDHHH (SEQ. ID. NO: 18)	HIPHH (SEQ. ID. NO: 24)			
Anabaena Δ^{12}	VLGHDCGH (SEQ. ID. NO: 8)	HNVHH (SEQ. ID. NO: 19)	HVPHH (SEQ. ID. NO: 25)			

*Pl-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arabidopsis Δ^{12} desaturase (fad2)

EXAMPLE 10

1 Construction of 222.1Δ⁶NOS for transient
and expression

5 The vector pBI221 (Jefferson et al. 1987
EMBO J. 6:3901-3907) was prepared for ligation by
digestion with BamHI and EcoICR I (Promega) which
excises the GUS coding region leaving the 35S promoter
and NOS terminator intact. The borage Δ 6-desaturase
10 cDNA was excised from the Bluescript plasmid
(Stratagene) by digestion with BamHI and XhoI. The
XhoI end was made blunt by use of the Klenow fragment.
This fragment was then cloned into the BamHI/EcoICR I
sites of pBI221, yielding 221.Δ⁶NOS (Fig. 7). In
15 221.Δ⁶.NOS, the remaining portion (backbone) of the
restriction map depicted in Fig. 7 is pBI221.

20

25

30

35

EXAMPLE 11

1

Construction of 121. Δ^6 .NOS for stable transformation

5 The vector pBI121 (Jefferson et al. 1987
EMBO J. 6:3901-3907) was prepared for ligation by
digestion with BamHI and EcoICR I (Promega) which
excises the GUS coding region leaving the 35S promoter
and NOS terminator intact. The borage Δ^6 -desaturase
cDNA was excised from the Bluescript plasmid
10 (Stratagene) by digestion with BamHI and XhoI. The
XhoI end was made blunt by use of the Klenow fragment.
This fragment was then cloned into the BamHI/EcoICR I
sites of pBI121, yielding 121.1 Δ^6 .NOS (Fig. 7). In
121. Δ^6 .NOS, the remaining portion (backbone) of the
15 restriction map depicted in Fig. 7 is pBI121.

20

25

30

35

EXAMPLE 12

Transient Expression

1
5 All work involving protoplasts was performed
in a sterile hood. One ml of packed carrot suspension
cells were digested in 30 mls plasmolyzing solution
(25 g/l KCl, 3.5 g/l $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 10mM MES, pH 5.6 and
0.2 M mannitol) with 1% cellulase, 0.1% pectolyase,
and 0.1% dreisalase overnight, in the dark, at room
10 temperature. Released protoplasts were filtered
through a 150 μm mesh and pelleted by centrifugation
(100x g, 5 min.) then washed twice in plasmolyzing
solution. Protoplasts were counted using a double
chambered hemocytometer. DNA was transfected into the
15 protoplasts by PEG treatment as described by Nunberg
and Thomas (1993 Methods in Plant Molecular Biology
and Biotechnology, B.R. Glick and J.E. Thompson, eds.
pp. 241-248) using 10^6 protoplasts and 50-70 ug of
plasmid DNA (221.Δ6.NOS). Protoplasts were cultured
20 in 5 mls of MS media supplemented with 0.2M mannitol
and 3 μm 2,4-D for 48 hours in the dark with shaking.

25

30

35

EXAMPLE 13

Stable transformation of tobacco

1
5 121.Δ⁶.NOS plasmid construction was used to
transform tobacco (*Nicotiana tabacum* cv. xanthi) via
Agrobacterium according to standard procedures (Horsh
et al., 1985 Science 227: 1229-1231; Bogue et al.,
1990 Mol. Gen. Genet. 221:49-57), except that initial
transformants were selected on 100 ug/ml kanamycin.

10

15

20

25

30

35

EXAMPLE 14

Preparation and analysis of
fatty acid methyl esters (FAMES)

1 Tissue from transfected protoplasts and
5 transformed tobacco plants was frozen in liquid
nitrogen and lyophilized overnight. FAMES were
prepared as described by Dahmer et al (1989 J. Amer.
Oil Chem. Soc. 66:543-548). In some cases, the
10 solvent was evaporated again, and the FAMES were
resuspended in ethyl acetate and extracted once with
deionized water to remove any water soluble
contaminants. The FAMES were analyzed by gas
chromatography (GC) on a J&W Scientific DB-wax column
(30 m length, 0.25 mm ID, 0.25 μ m film).

15 An example of a transient assay is shown in
Fig. 8 which represents three independent
transfections pooled together. The addition of the
borage $\Delta 6$ -desaturase cDNA corresponds with the
appearance of gamma linolenic acid (GLA) which is one
20 of the possible products of $\Delta 6$ -desaturase.

Figures 9 and 10 depict GC profiles of the
FAMES derived from leaf and seed tissue, respectively,
of control and transformed tobacco plants. Figure 9A
provides the profile of leaf tissue of wild-type
25 tobacco (xanthi); Figure 9B provides the profile of
leaf tissue from a tobacco plant transformed with the
borage Δ -6 desaturase under the transcriptional
control of the 35S CaMV promoter (pBI 121 Δ 'NOS).
Peaks correspond to 18:2, 18:3 γ (GLA), 18:3 α and 18:4
30 (octadecanonic acid). Figure 10A shows the GC profile
of seeds of a wild-type tobacco; Figure 10B shows the

- 1 profile of seed tissue of a tobacco plant transformed
with pBI 121 Δ^6 NOS. Peaks correspond to 18:2,
18:3 γ (GLA) and 18:3 α .

- 5 The relative distribution of the C₁₈ fatty
acids in control and transgenic tobacco seeds is shown
in Table 4.

TABLE 4

	Fatty Acid	Xanthi	pBI121 Δ^6 NOS
10	18:0	4.0%	2.5%
	18:1	13%	13%
	18:2	82%	82%
	18:3 γ (GLA)	-	2.7%
15	18:3 α	0.82%	1.4%

- 20 The foregoing results demonstrate that GLA
is incorporated into the triacylglycerides of
transgenic tobacco leaves and seeds containing the
borage Δ^6 -desaturase.

25

30

35

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Rhone-Poulenc Agrochimie
- (ii) TITLE OF INVENTION: PRODUCTION OF GAMMA LINOLENIC ACID BY A DELTA 6-DESATURASE
- (iii) NUMBER OF SEQUENCES: 25
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Scully, Scott, Murphy & Presser
 - (B) STREET: 400 Garden City Plaza
 - (C) CITY: Garden City
 - (D) STATE: New York
 - (E) COUNTRY: United States
 - (F) ZIP: 11530
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 30-DEC-1994
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Presser, Leopold
 - (B) REGISTRATION NUMBER: 19,827
 - (C) REFERENCE/DOCKET NUMBER: 8383ZYXW
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (516) 742-4343
 - (B) TELEFAX: (516) 742-4366
 - (C) TELEX: 230 901 SANS UR

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3588 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 2002..3081

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTAGCCACC AGTGACGATG CCTTGAATTT GGCCATTCTG ACCCAGGCCC GTATTCTGAA	60
TCCCCGCATT CGCATTGTTA ATCGTTTGTT CAACCATGCC CTGGGTAAAC GTTTAGACAC	120
CACCTTGCCA GACCACGTTA GTTTGAGTGT TTCCGCCCTG GCGGCCCCGA TTTTTTCCTT	180
TGCGGCTTTG GGCAATCAGG CGATCGGGCA ATTGCGTTTG TTTGACCAGA CTGGGCCCAT	240
TCAGGAAATT GTCATTCACC AAGACCATCC CTGGCTCAAT TTACCCCTGG CGGATTTATG	300
GGATGATCCG AGCCGAATGT TGATCTATTA CCTACCGGCC CACAGTGAAA CGGATTTAGT	360
AGGCGCAGTG GTGAATAATT TAACGTTGCA ATCTGGGGAC CATTTAATAG TGGGACAAAA	420
ACCCCAACCC AAGACCAAAC GGCATCGCC TTGGCGCAA TTTTCCAAAC TGATTACCAA	480
CCTGCGGGAG TATCAGCGGT ATGTCCAACA GGTGATATGG GTGGTGTGT TTTTATTGTT	540
GATGATTTTT CTGGCCACCT TCATCTACGT TTCCATTGAT CAACATATTG CCCCAGTGGA	600
CGCGTTGTAT TTTTCCGTGG GCATGATTAC CGGGGCCGGT GGCAAGGAAG AGGTGGCCGA	660
AAAGTCCCCC GATATCATCA AAGTATTCAC AGTGGTGATG ATGATCGCCG GGGCGGGGGT	720
GATTGGTATT TGTTATGCCC TACTGAATGA TTTCATCCTT GGCAGTCGCT TTAGTCAGTT	780
TTTGGATGCG GCCAAGTTAC CCGATCGCCA TCACATCATC ATTTGTGGGC TGGGGGGAGT	840
GAGCATGGCC ATTATTGAAG AGTTAATTCA CCAGGGCCAT GAAATTGTGG TAATCGAAAA	900
GGATACAGAT AATCGTTTCT TGCATACGGC CCGCTCCCTG GGGGTGCCCC TAATTGTGGA	960
GGATGCCCCG CTAGAAAGAA CGTTGGCCTG CGCCAATATC AACCGAGCCG AAGCCATTGT	1020
GGTGGCCACC AGCGACGACA CCGTTAACTT GGAAATTGGC CTAAGTGCCA AGGCGATCGC	1080
CCCTAGCCTG CCAGTGGTGT TCGTTTGCCA GGATGCCCAG TTTAGCCTGT CCCTGCAGGA	1140
AGTATTTGAA TTTGAAACGG TGCTTTGTCC GGCAGGAATTG GCCACCTATT CCTTTGCGGC	1200
GGCGGCCCTG GGGGGCAAAA TTTTGGGCAA CGGCATGACC GATGATTGTC TGTGGGTAGC	1260
CCTAGCCACC TTAATCACTC CTAACCATCC CTTTGCCGAC CAATTGGTTA AAATTGCAGC	1320
CCAAAAGTCT GATTTTCGTT CCCTCTATCT AGAACGGGGT GGCAAAACCA TCCATAGCTG	1380
GGAATTATTG GGTACCCATC TCGACTCTGG AGACGTGTTG TATTTAACCA TGCCCGCCAC	1440
TGCCCTAGAG CAACTTTGGC GATCGCCCCG TGCCACTGCT GATCCTCTGG ACTCTTTTTT	1500

GGTTTAGCAT GGGGGGATGG AACTCTTGAC TCGGCCCAAT GGTGATCAAG AAAGAACGCT 1560
TTGTCTATGT TTAGTATTTT TAAGTTAACC AACAGCAGAG GATAACTTCC AAAAGAAATT 1620
AAGCTCAAAA AGTAGCAAAA TAAGTTTAAT TCATAACTGA GTTTTACTGC TAAACAGCGG 1680
TGCAAAAAAG TCAGATAAAA TAAAAGCTTC ACTTCGGTTT TATATTGTGA CCATGGTTCC 1740
CAGGCATCTG CTCTAGGGAG TTTTCCGCT GCCTTTAGAG AGTATTTTCT CCAAGTCGGC 1800
TAACTCCCCC ATTTTtagGC AAAATCATAT ACAGACTATC CCAATATTGC CAGAGCTTTG 1860
ATGACTCACT GTAGAAGGCA GACTAAAATT CTAGCAATGG ACTCCCAGTT GGAATAAATT 1920
TTTAGTCTCC CCCGGCGCTG GAGTTTTTTT GTAGTTAATG GCGGTATAAT GTGAAAGTTT 1980
TTTATCTATT TAAATTTATA A ATG CTA ACA GCG GAA AGA ATT AAA TTT ACC 2031
Met Leu Thr Ala Glu Arg Ile Lys Phe Thr
1 5 10
CAG AAA CGG GGG TTT CGT CGG GTA CTA AAC CAA CGG GTG GAT GCC TAC 2079
Gln Lys Arg Gly Phe Arg Arg Val Leu Asn Gln Arg Val Asp Ala Tyr
15 20 25
TTT GCC GAG CAT GGC CTG ACC CAA AGG GAT AAT CCC TCC ATG TAT CTG 2127
Phe Ala Glu His Gly Leu Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu
30 35 40
AAA ACC CTG ATT ATT GTG CTC TGG TTG TTT TCC GCT TGG GCC TTT GTG 2175
Lys Thr Leu Ile Ile Val Leu Trp Leu Phe Ser Ala Trp Ala Phe Val
45 50 55
CTT TTT GCT CCA GTT ATT TTT CCG GTG CGC CTA CTG GGT TGT ATG GTT 2223
Leu Phe Ala Pro Val Ile Phe Pro Val Arg Leu Leu Gly Cys Met Val
60 65 70
TTG GCG ATC GCC TTG GCG GCC TTT TCC TTC AAT GTC GGC CAC GAT GCC 2271
Leu Ala Ile Ala Leu Ala Ala Phe Ser Phe Asn Val Gly His Asp Ala
75 80 85 90
AAC CAC AAT GCC TAT TCC TCC AAT CCC CAC ATC AAC CGG GTT CTG GGC 2319
Asn His Asn Ala Tyr Ser Ser Asn Pro His Ile Asn Arg Val Leu Gly
95 100 105
ATG ACC TAC GAT TTT GTC GGG TTA TCT AGT TTT CTT TGG CGC TAT CGC 2367
Met Thr Tyr Asp Phe Val Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg
110 115 120
CAC AAC TAT TTG CAC CAC ACC TAC ACC AAT ATT CTT GGC CAT GAC GTG 2415
His Asn Tyr Leu His His Thr Tyr Thr Asn Ile Leu Gly His Asp Val
125 130 135
GAA ATC CAT GGA GAT GGC GCA GTA CGT ATG AGT CCT GAA CAA GAA CAT 2463
Glu Ile His Gly Asp Gly Ala Val Arg Met Ser Pro Glu Gln Glu His
140 145 150

GTT GGT ATT TAT CGT TTC CAG CAA TTT TAT ATT TGG GGT TTA TAT CTT Val Gly Ile Tyr Arg Phe Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu 155 160 165 170	2511
TTC ATT CCC TTT TAT TGG TTT CTC TAC GAT GTC TAC CTA GTG CTT AAT Phe Ile Pro Phe Tyr Trp Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn 175 180 185	2559
AAA GGC AAA TAT CAC GAC CAT AAA ATT CCT CCT TTC CAG CCC CTA GAA Lys Gly Lys Tyr His Asp His Lys Ile Pro Pro Phe Gln Pro Leu Glu 190 195 200	2607
TTA GCT AGT TTT CTA GGG ATT AAG CTA TTA TGG CTC GGC TAC GTT TTC Leu Ala Ser Leu Leu Gly Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe 205 210 215	2655
GGC TTA CCT CTG GCT CTG GGC TTT TCC ATT CCT GAA GTA TTA ATT GGT Gly Leu Pro Leu Ala Leu Gly Phe Ser Ile Pro Glu Val Leu Ile Gly 220 225 230	2703
GCT TCG GTA ACC TAT ATG ACC TAT GGC ATC GTG GTT TGC ACC ATC TTT Ala Ser Val Thr Tyr Met Thr Tyr Gly Ile Val Val Cys Thr Ile Phe 235 240 245 250	2751
ATG CTG GCC CAT GTG TTG GAA TCA ACT GAA TTT CTC ACC CCC GAT GGT Met Leu Ala His Val Leu Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly 255 260 265	2799
GAA TCC GGT GCC ATT GAT GAC GAG TGG GCT ATT TGC CAA ATT CGT ACC Glu Ser Gly Ala Ile Asp Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr 270 275 280	2847
ACG GCC AAT TTT GCC ACC AAT AAT CCC TTT TGG AAC TGG TTT TGT GGC Thr Ala Asn Phe Ala Thr Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly 285 290 295	2895
GGT TTA AAT CAC CAA GTT ACC CAC CAT CTT TTC CCC AAT ATT TGT CAT Gly Leu Asn His Gln Val Thr His His Leu Phe Pro Asn Ile Cys His 300 305 310	2943
ATT CAC TAT CCC CAA TTG GAA AAT ATT ATT AAG GAT GTT TGC CAA GAG Ile His Tyr Pro Gln Leu Glu Asn Ile Ile Lys Asp Val Cys Gln Glu 315 320 325 330	2991
TTT GGT GTG GAA TAT AAA GTT TAT CCC ACC TTC AAA GCG GCG ATC GCC Phe Gly Val Glu Tyr Lys Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala 335 340 345	3039
TCT AAC TAT CGC TGG CTA GAG GCC ATG GGC AAA GCA TCG TGACATTGCC Ser Asn Tyr Arg Trp Leu Glu Ala Met Gly Lys Ala Ser 350 355 360	3088
TTGGGATTGA AGCAAAATGG CAAAATCCCT CGTAAATCTA TGATCGAAGC CTTTCTGTTG	3148
CCCGCCGACC AAATCCCCGA TGCTGACCAA AGGTTGATGT TGGCATTGCT CCAAACCCAC	3208

TTTGAGGGGG TTCATTGGCC GCAGTTTCAA GCTGACCTAG GAGGCAAAGA TTGGGTGATT 3268
 TTGCTCAAAT CCGCTGGGAT ATTGAAAGGC TTCACCACCT TTGGTTTCTA CCCTGCTCAA 3328
 TGGGAAGGAC AAACCGTCAG AATTGTTTAT TCTGGTGACA CCATCACC GA CCCATCCATG 3388
 TGGTCTAACC CAGCCCTGGC CAAGGCTTGG ACCAAGGCCA TGCAAATTCT CCACGAGGCT 3448
 AGGCCAGAAA AATTATATTG GCTCCTGATT TCTTCCGGCT ATCGCACCTA CCGATTTTGT 3508
 AGCATTTTTG CCAAGGAATT CTATCCCCAC TATCTCCATC CCACTCCCCC GCCTGTACAA 3568
 AATTTTATCC ATCAGCTAGC 3588

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 359 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg
 1 5 10 15
 Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu
 20 25 30
 Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val
 35 40 45
 Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile
 50 55 60
 Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala
 65 70 75 80
 Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser
 85 90 95
 Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val
 100 105 110
 Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His
 115 120 125
 Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly
 130 135 140
 Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe
 145 150 155 160

[illegible]

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1884 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGCTTCACCTT CGGTTTTATA TTGTGACCAT GGTTCACAGG CATCTGCTCT AGGGAGTTTT 60
TCCGCTGCCT TTAGAGAGTA TTTTCTCCAA GTCGGCTAAC TCCCCATTT TTAGGCAAAA 120

TCATATACAG ACTATCCCAA TATTGCCAGA GCTTTGATGA CTCACTGTAG AAGGCAGACT	180
AAAATTCTAG CAATGGACTC CCAGTTGGAA TAAATTTTTA GTCTCCCCCG GCGCTGGAGT	240
TTTTTTGTAG TTAATGGCGG TATAATGTGA AAGTTTTTTA TCTATTTAAA TTTATAAATG	300
CTAACAGCGG AAAGAATTAA ATTTACCCAG AAACGGGGGT TTCGTCGGGT ACTAAACCAA	360
CGGGTGGATG CCTACTTTGC CGAGCATGGC CTGACCCAAA GGGATAATCC CTCCATGTAT	420
CTGAAAACCC TGATTATTGT GCTCTGGTTG TTTCCGCTT GGGCCTTTGT GCTTTTTGCT	480
CCAGTTATTT TTCCGGTGCG CCTACTGGGT TGTATGGTTT TGGCGATCGC CTTGGCGGCC	540
TTTTCTTCA ATGTCGGCCA CGATGCCAAC CACAATGCCT ATTCCTCCAA TCCCCACATC	600
AACCGGGTTC TGGGCATGAC CTACGATTTT GTCGGGTAT CTAGTTTTCT TTGGCGCTAT	660
CGCCACAACCT ATTTGCACCA CACCTACACC AATATTCTTG GCCATGACGT GGAAATCCAT	720
GGAGATGGCG CAGTACGTAT GAGTCCTGAA CAAGAACATG TTGGTATTTA TCGTTTCCAG	780
CAATTTTATA TTTGGGGTTT ATATCTTTTC ATTCCCTTTT ATTGGTTTCT CTACGATGC	840
TACCTAGTGC TTAATAAAGG CAAATATCAC GACCATAAAA TTCCTCCTTT CCAGCCCCCTA	900
GAATTAGCTA GTTTGCTAGG GATTAAGCTA TTATGGCTCG GCTACGTTTT CGGCTTACCT	960
CTGGCTCTGG GCTTTTCCAT TCCTGAAGTA TTAATTGGTG CTTCGGTAAC CTATATGACC	1020
TATGGCATCG TGGTTTGCAC CATCTTTATG CTGGCCCATG TGTTGGAATC AACTGAATTT	1080
CTACCCCCG ATGGTGAATC CGGTGCCATT GATGACGAGT GGGCTATTTG CCAAATTCGT	1140
ACCACGGCCA ATTTTGCCAC CAATAATCCC TTTTGGAAC TGGTTTGTGG CGGTTTAAAT	1200
CACCAAGTTA CCCACCATCT TTTCCCCAAT ATTTGTCATA TTCACTATCC CCAATTGGAA	1260
AATATTATTA AGGATGTTTG CCAAGAGTTT GGTGTGGAAT ATAAAGTTTA TCCCACCTTC	1320
AAAGCGGCGA TCGCCTCTAA CTATCGCTGG CTAGAGGCCA TGGGCAAAGC ATCGTGACAT	1380
TGCCTTGGGA TTGAAGCAAA ATGGCAAAAT CCCTCGTAAA TCTATGATCG AAGCCTTTCT	1440
GTTGCCCGCC GACCAAATCC CCGATGCTGA CCAAAGTTG ATGTTGGCAT TGCTCCAAAC	1500
CCACTTTGAG GGGGTTTATT GGCCGCAGTT TCAAGCTGAC CTAGGAGGCA AAGATTGGGT	1560
GATTTTGCTC AAATCCGCTG GGATATTGAA AGGCTTCACC ACCTTTGGTT TCTACCCTGC	1620
TCAATGGGAA GGACAAACCG TCAGAATTGT TTATTCTGGT GACACCATCA CCGACCCATC	1680
CATGTGGTCT AACCAGCCCC TGGCCAAGGC TTGGACCAAG GCCATGCAAA TTCTCCACGA	1740
GGCTAGGCCA GAAAAATTAT ATGGCTCCT GATTTCTTCC GGCTATCGCA CCTACCGATT	1800

TTTGAGCATT TTGCCAAGG AATTCTATCC CCACTATCTC CATCCCACTC CCCC GCCTGT 1860
 ACAAATTTT ATCCATCAGC TAGC 1884

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1685 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATATCTGCC TACCCTCCCA AAGAGAGTAG TCATTTTTC TCAATGGCTG CTCAAATCAA 60
 GAAATACATT ACCTCAGATG AACTCAAGAA CCACGATAAA CCCGGAGATC TATGGATCTC 120
 GATTCAAGGG AAAGCCTATG ATGTTTCGGA TTGGGTGAAA GACCATCCAG GTGGCAGCTT 180
 TCCCTTGAAG AGTCTTGCTG GTCAAGAGGT AACTGATGCA TTTGTTGCAT TCCATCCTGC 240
 CTCTACATGG AAGAATCTTG ATAAGTTTTT CACTGGGTAT TATCTTAAAG ATTACTCTGT 300
 TTCTGAGGTT TCTAAAGATT ATAGGAAGCT TGTGTTTGAG TTTTCTAAAA TGGGTTTGTA 360
 TGACAAAAAA GGTCAATATTA TGTTTGCAAC TTTGTGCTTT ATAGCAATGC TGTTTGCTAT 420
 GAGTGTTTAT GGGGTTTTGT TTTGTGAGGG TGTTTGGTA CATTTGTTTT CTGGGTGTTT 480
 GATGGGGTTT CTTTGGATTC AGAGTGGTTG GATTGGACAT GATGCTGGGC ATTATATGGT 540
 AGTGTCTGAT TCAAGGCTTA ATAAGTTTAT GGGTATTTTT GCTGCAAATT GTCTTTCAGG 600
 AATAAGTATT GGTGGTGGA AATGGAACCA TAATGCACAT CACATTGCCT GTAATAGCCT 660
 TGAATATGAC CCTGATTTAC AATATATACC ATTCCTTGTT GTGTCTTCCA AGTTTTTTGG 720
 TTCCTCACC TCTCATTTCT ATGAGAAAAG GTTGACTTTT GACTCTTTAT CAAGATTCTT 780
 TGTAAGTTAT CAACATTGGA CATTTTACCC TATTATGTGT GCTGCTAGGC TCAATATGTA 840
 TGTACAATCT CTCATAATGT TGTGACCAA GAGAAATGTG TCCTATCGAG CTCAGGAACT 900
 CTGGGGATGC CTAGTGTCT CGATTTGGTA CCCGTTGCTT GTTCTTGTT TGCCTAATTG 960
 GGGTGAAAGA ATTATGTTTG TTATTGCAAG TTTATCAGTG ACTGGAATGC AACAAGTTCA 1020
 GTTCTCCTTG AACCACTTCT CTTCAAGTGT TTATGTTGGA AAGCCTAAAG GGAATAATTG 1080
 GTTTGAGAAA CAAACGGATG GGACACTTGA CATTTCTTGT CCTCCTTGGA TGGATTGGTT 1140
 TCATGGTGGA TTGCAATTCC AAATTGAGCA TCATTTGTTT CCCAAGATGC CTAGATGCAA 1200

```

CCTTAGGAAA ATCTCGCCCT ACGTGATCGA GTTATGCAAG AAACATAATT TGCCTTACAA      1260
TTATGCATCT TTCTCCAAGG CCAATGAAAT GACACTCAGA ACATTGAGGA ACACAGCATT      1320
GCAGGCTAGG GATATAACCA AGCCGCTCCC GAAGAATTTG GTATGGGAAG CTCTTCACAC      1380
TCATGGTTAA AATTACCCTT AGTTCATGTA ATAATTTGAG ATTATGTATC TCCTATGTTT      1440
GTGTCTTGTC TTGGTTCTAC TTGTTGGAGT CATTGCAACT TGTCTTTTAT GGTTTATTAG      1500
ATGTTTTTTA ATATATTTTA GAGGTTTTGC TTTCATCTCC ATTATTGATG AATAAGGAGT      1560
TGCATATTGT CAATTGTTGT GCTCAATATC TGATATTTTG GAATGTACTT TGTACCACTG      1620
TGTTTTTCAGT TGAAGCTCAT GTGTACTTCT ATAGACTTTG TTAAATGGT TATGTCATGT      1680
TATTTT                                     1685

```

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 448 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
 1           5           10
His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr
 20          25          30
Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
 35          40          45
Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
 50          55          60
Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
 65          70          75
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
 85          90          95
Val Phe Glu Phe Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile
100         105         110
Met Phe Ala Thr Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val
115         120         125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly
130         135         140

```

Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp
 145 150 155 160
 Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met
 165 170 175
 Gly Ile Phe Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
 180 185 190
 Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr
 195 200 205
 Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe
 210 215 220
 Phe Gly Ser Leu Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp
 225 230 235 240
 Ser Leu Ser Arg Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro
 245 250 255
 Ile Met Cys Ala Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met
 260 265 270
 Leu Leu Thr Lys Arg Asn Val Ser Tyr Arg Ala Gln Glu Leu Leu Gly
 275 280 285
 Cys Leu Val Phe Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
 290 295 300
 Asn Trp Gly Glu Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr
 305 310 315 320
 Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val
 325 330 335
 Tyr Val Gly Lys Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp
 340 345 350
 Gly Thr Leu Asp Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly
 355 360 365
 Gly Ser Gln Phe Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg
 370 375 380
 Cys Asn Leu Arg Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys
 385 390 395 400
 His Asn Leu Pro Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met
 405 410 415
 Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
 420 425 430
 Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly
 435 440 445

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Trp Ile Gly His Asp Ala Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Val Gly His Asp Ala Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Leu Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

-55-

Val Ile Ala His Glu Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Ile Gly His Asp Cys Ala His
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Val Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

His Asn Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

-56-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

His Asn Tyr Leu His His
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

His Arg Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Arg Arg His His
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Asp Arg His His
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Asp Gln His His
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Asp His His His
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-58-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Asn His His His
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Phe Gln Ile Glu His His
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

His Gln Val Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His Val Ile His His
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid

-59-

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Val Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

His Ile Pro His His
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

His Val Pro His His
1 5

1 WHAT IS CLAIMED:

1. An isolated nucleic acid encoding a borage $\Delta 6$ -desaturase.

5 2. The isolated nucleic acid of Claim 1 comprising the nucleotide sequence of SEQ ID NO: 4.

10 3. An isolated nucleic acid that codes for the amino acid sequence of SEQ ID NO: 5.

4. A vector comprising the nucleic acid of any one Claims 1-3.

15 5. An expression vector comprising the isolated nucleic acid of any one of Claims 1-3 operably linked to a promoter and optionally a termination signal capable of effecting expression of the gene product of said isolated nucleic acid.

20 6. The expression vector of Claim 5 wherein said promoter is a Δ -6 desaturase promoter, an Anabaena carboxylase promoter, a helianthinin promoter, a glycinin promoter, a napin promoter, the 35S promoter from CaMV, or
25 a helianthinin tissue-specific promoter.

7. The expression vector of Claim 5 wherein said promoter is constitutive or tissue-specific.

30 8. The expression vector of Claim 5 wherein said termination signal is a Synechocystis termination

1 signal, a nopaline synthase termination signal, or a seed
termination signal.

5 9. A cell comprising the vector of any one of
Claims 4-8.

10 10. The cell of Claim 9 wherein said cell is an
animal cell, a bacterial cell, a plant cell or a fungal
cell.

11. A transgenic organism comprising the
isolated nucleic acid of any one of Claims 1-3.

15 12. A transgenic organism comprising the vector
of any one of Claims 4-8.

20 13. The transgenic organism of Claim 11 or 12
wherein said organism is a bacterium, a fungus, a plant or
an animal.

25 14. A plant or progeny of said plant which has
been regenerated from the plant cell of Claim 10.

30 15. The plant of Claim 14 wherein said plant is
a sunflower, soybean, maize, tobacco, peanut, carrot or
oil seed rape plant.

35 16. A method of producing a plant with
increased gamma linolenic acid (GLA) content which
comprises:

1 (a) transforming a plant cell with the isolated
nucleic acid of any one of Claims 1-3; and

(b) regenerating a plant with increased GLA
content from said plant cell.

5

17. A method of producing a plant with
increased gamma linolenic acid (GLA) content which
comprises:

(a) transforming a plant cell with the vector of
10 any one of Claims 4-8; and

(b) regenerating a plant with increased GLA
content from said plant cell.

15 18. The method of Claim 16 or 17 wherein said
plant is a sunflower, soybean, maize, tobacco, peanut,
carrot or oil seed rape plant.

19. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
20 in GLA which comprises transforming said organism with the
isolated nucleic acid of any one of Claims 1-3.

20. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
25 in GLA which comprises transforming said organism with the
vector of any one of Claims 4-8.

21. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
30 in GLA and linoleic acid (LA) which comprises transforming
said organism with an isolated nucleic acid encoding

35

- 1 borage $\Delta 6$ -desaturase and an isolated nucleic acid encoding
12-desaturase.

22. The method of Claim 21 wherein said
5 isolated nucleic acid encoding $\Delta 6$ -desaturase comprises
nucleotides 44 to 1390 of SEQ. ID NO: 4.

23. A method of inducing production of
octadecatetraeonic acid in an organism deficient or
10 lacking in gamma linolenic acid which comprises
transforming said organism with the isolated nucleic acid
of any one of Claims 1-3.

24. A method of inducing production of
15 octadecatetraeonic acid in an organism deficient or
lacking in gamma linolenic acid which comprises
transforming said organism with the vector of any one of
Claims 4-8.

- 20 25. The method of Claim 23 or 24 wherein said
organism is a bacterium, a fungus, a plant or an animal.

26. A method of producing a plant with improved
chilling resistance which comprises:

- 25 (a) transforming a plant cell with the isolated
nucleic acid of any one of Claims 1-3; and
(b) regenerating said plant with improved
chilling resistance from said transformed plant cell.

- 30 27. A method of producing a plant with improved
chilling resistance which comprises:

35

1 (a) transforming a plant cell with the vector of
any one of Claims 4-8; and

(b) regenerating said plant with improved
chilling resistance from said transformed plant cell.

5 28. The method of Claim 26 or 27 wherein said
plant is a sunflower, soybean, maize, tobacco, peanut,
carrot or oil seed rape plant.

10

15

20

25

30

35

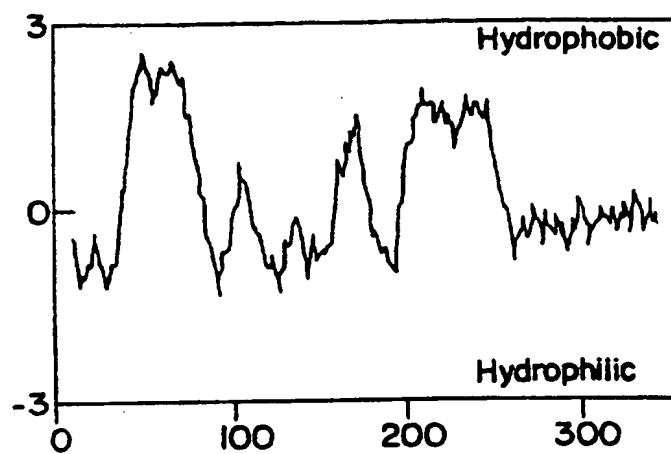


FIG. IA

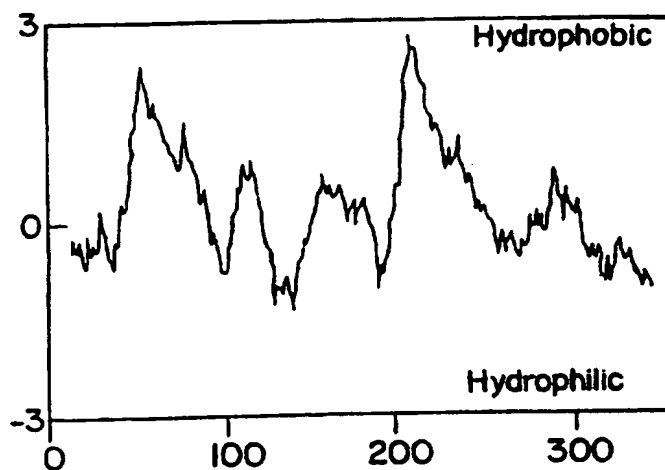
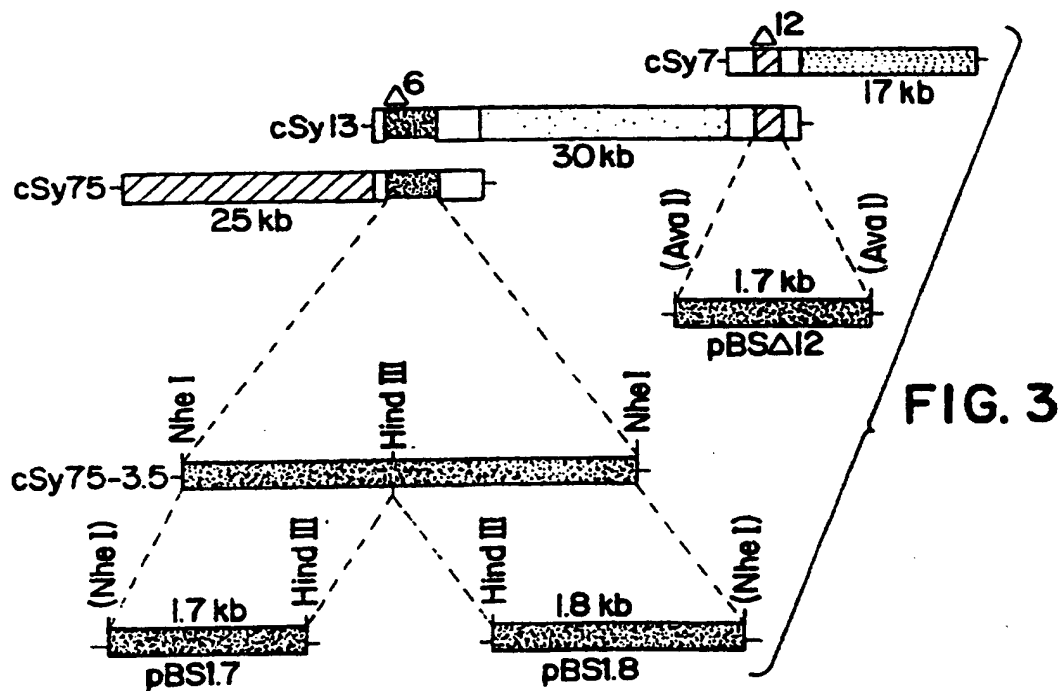
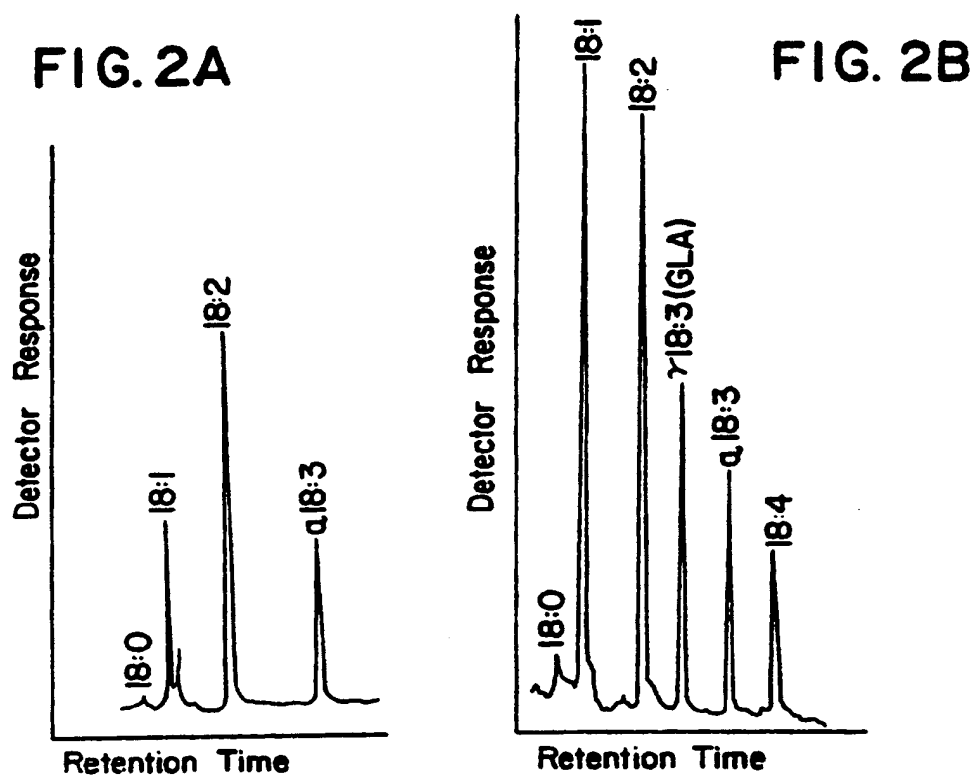


FIG. IB

2 / 9



SUBSTITUTE SHEET (RULE 26)

3 / 9

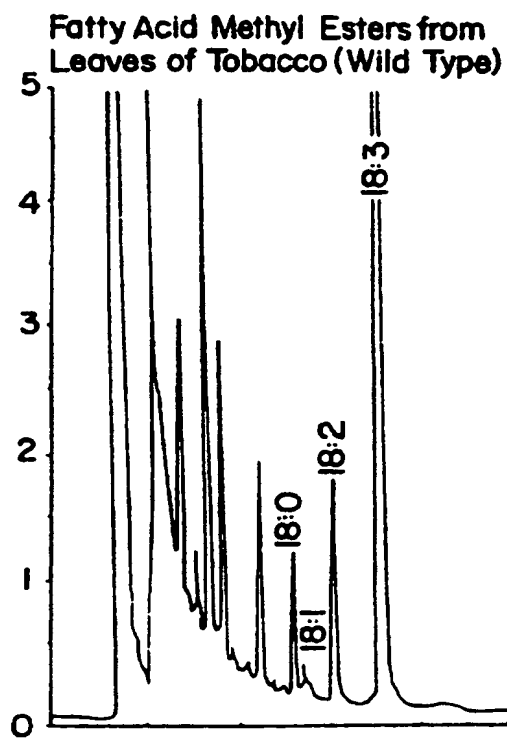


FIG. 4A

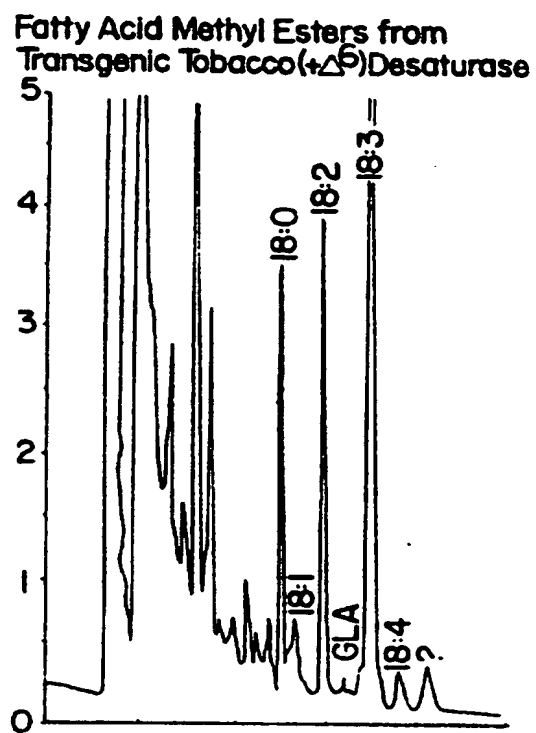


FIG. 4B

4 / 9

FIG.5A

1 aatatctgcc taccctccca aagagagtag tcatcttttca tcaatggctg ctcaaatcaa gaaatacatt acctcagatg 80
 81 aactcaagaa ccacgataaa cccggagatc tatggatctc gattcaaggg aagcctatg atgtttcggg ttgggtgaaa 160
 161 gaccatccag gtggcagctt tcccttgaag agtcttgctg gtcaagaggt aactgatgca ttgtttgcat tccatcctgc 240
 241 ctctacatgg aagaatcttg ataatgtttt cactgggtat tatcttaag attactctgt ttctgaggtt tctaaagatt 320
 321 ataggaagct tgtgtttgag ttttctaaaa tgggttttga tgacaaaaa ggtcatatta tgtttgcaac ttgtgctttt 400
 401 atagcaatgc tgtttgctat gagtgtttat ggggtttttg tttgtgaggg tgttttggtt cattttgtttt ctgggtgctt 480
 481 gatgggggtt ctttggattc agagtgggtt gattggacat gatgctgggc attatatggt agtgctctgat tcaaggctta 560
 561 ataatgtttat ggttattttt gctgcaaat gcttttcagg aataagtatt ggttggtgga aatggaacca taatgcacat 640
 641 cacattgcct gtaatagcct tgaatatgac cctgattttac aatatatacc attccttgtt gtgtcttcca agttttttgg 720
 721 ttcactcacc tctcatttct atgagaaaaa gttgactttt gactctttat caagattctt tgaagtatat caacattgga 800
 801 cattttacc tattatgtgt gctgctaggc tcaatatgta tgtacaatct ctcataatgt tgttgaccaa gagaaatgtg 880
 881 tccctatogag ctccaggact cttgggatgc ctatgtttct cgatttggtt cccgttgctt gtttcttgtt tgcctaattg 960
 961 ggggtgaaga attatgtttg ttattgcaag ttatcagtg actggaatgc aacaagtcca gttctccttg aaccttctt 1040
 1041 ctccaagtgt ttatgttga aagcctaaag ggaataattg gtttgagaaa caaacggatg ggaacattga catttcttct 1120
 1121 cctccttga tggatgggtt tcatggtgga ttgcaattcc aaattgagca tcaatttgtt cccaagatgc ctatgtgcaa 1200
 1201 ccttaggaaa atctgcctc acgtgatcga gttatgcaag aacataatt tgccttaca ttatgcatct tctccaagg 1280
 1281 ccaatgaat gacactcaga acattgagga acacagactt gcaggtagg gataatacca agccgtctcc gaagaatttg 1360
 1361 gtatgggaag ctcttcacac tcatggttaa aattaccctt agttcatgta ataatgttag attatgtatc tcttatgttt 1440
 1441 gtgtcttgc ttggttctac ttgttggagt cattgcaact tgccttttat ggtttatgag atgtttttta atatatatta 1520
 1521 gaggttttgc ttcatctcc attattgatg aataaaggat tgcataattgt caattgtgt gctcaaatatc tgatatatttg 1600
 1601 gaatgtactt tgtaccactg tgttttccagt tgaagctcat gtgtacttct atagactttg tttaaatggt tatgtcatgt 1680
 1681 tattt 1685

FIG.5B

1 MAAQIKKYIT SDELKNHDKP GDLWISIQGK AYDVSDMWKD HPGGSFPLKS LAGQEVTD AF VAFHPASTWK NLDKFFFTGY 80
 81 LKDYSVSEVS KDYRKLVEEF SKMGLYDKKG HIMFATLCFI AMLFAMSVYG VLFCEGVLVH LFSGCLMGFL WIQSGWIGHD 160
 161 AGHYMVVSDS RLNKFMGIFA ANCLSGISIG WWKWNHNAHH IACNSLEYDP DLQYIPFLVV SSKFFGSLTS HFYEKRLTFD 240
 241 SLSRFFVSQY HWTFPIMCA ARLNMVQSL IMLLTKRNVV YRAQELLGCL VFSIWYPLLV SCLPNWGERI MFVIASLSVT 320
 321 GMOQVQFSLN HFSSSVYVGK PKGNWFEKQ TDGTLDISCP PMDWFHGG L QFQIEHHLP KMPRCNLRKI SPYVIELCKK 400
 401 HNLPLYNASE SKANEMTLRT LRNTALQARD ITKPLPKNLV WEALHTHG 448

FIG. 6

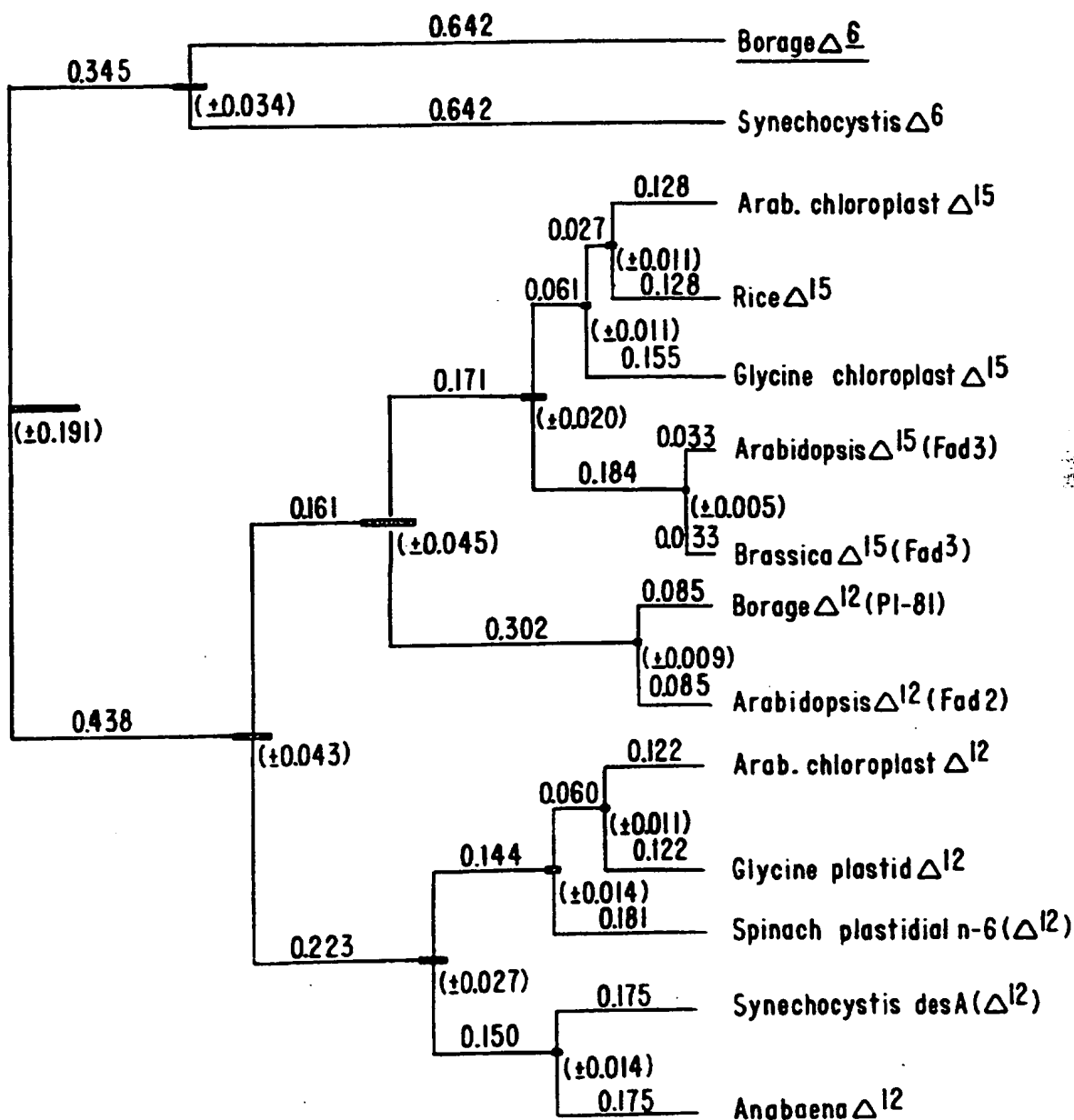


FIG. 7

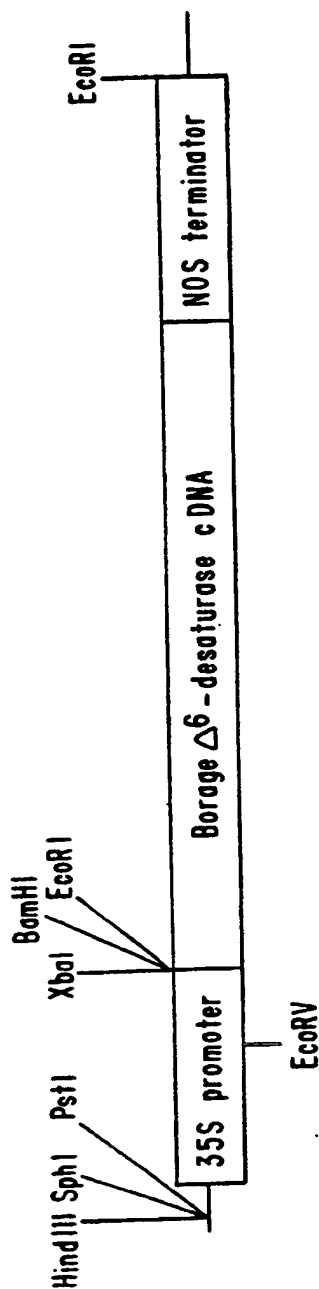


FIG. 8A

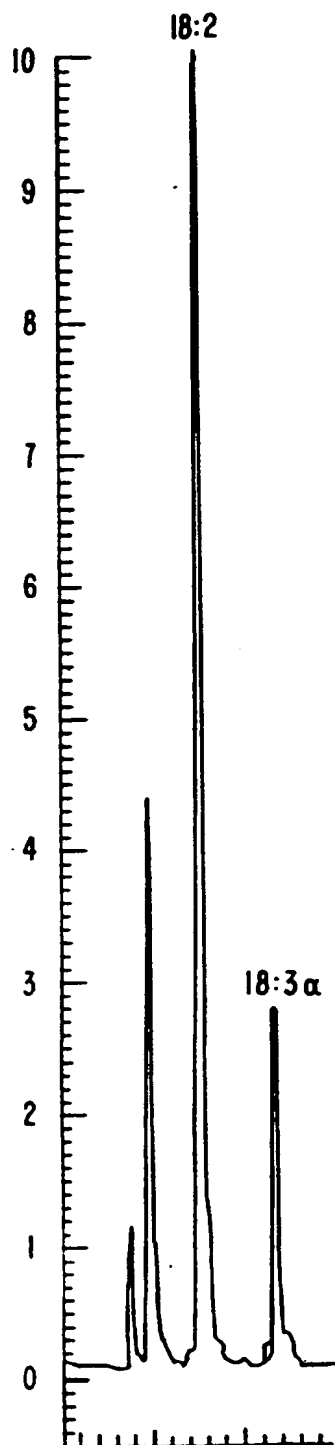


FIG. 8B

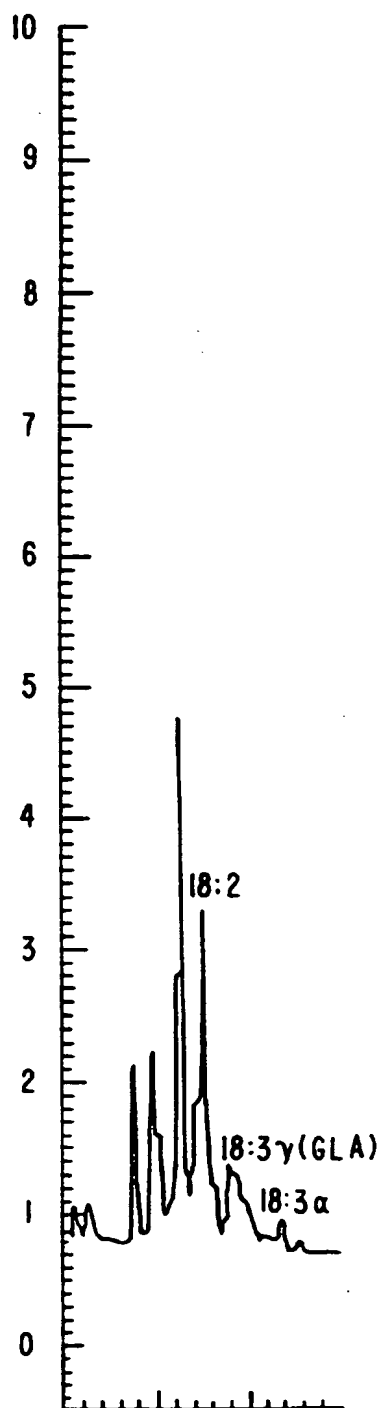


FIG. 9B

FIG. 9A

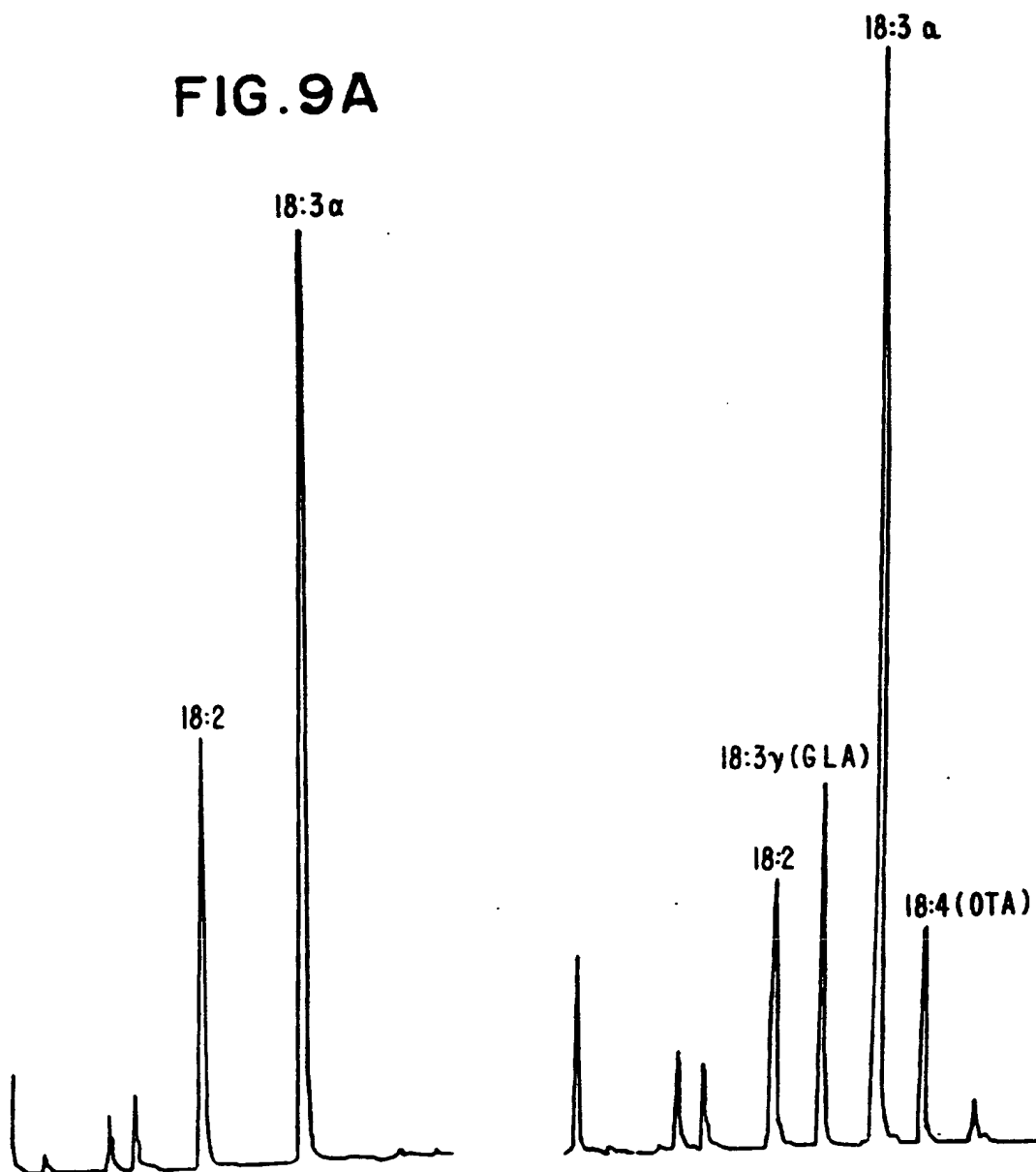


FIG. 10A

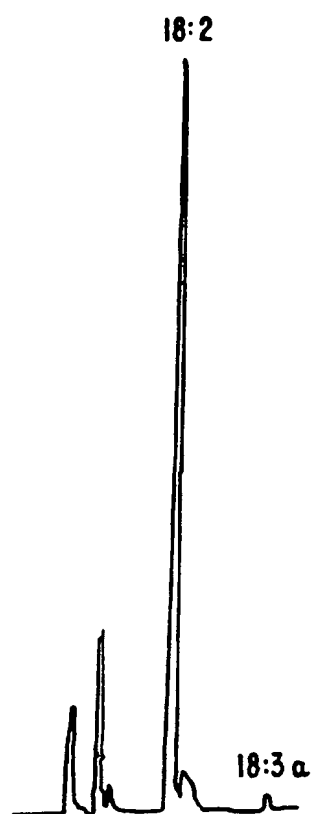
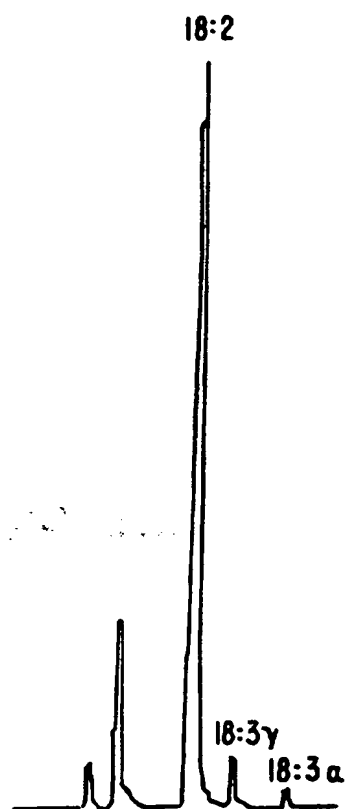


FIG. 10B



This Page Blank (uspto)

This Page Blank (uspto)

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/53, 15/82, A01H 5/00	A3	(11) International Publication Number: WO 96/21022 (43) International Publication Date: 11 July 1996 (11.07.96)
(21) International Application Number: PCT/IB95/01167 (22) International Filing Date: 28 December 1995 (28.12.95) (30) Priority Data: 08/366,779 30 December 1994 (30.12.94) US (71) Applicant: RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre-Baizet, F-69263 Lyon (FR). (72) Inventors: THOMAS, Terry, L.; 3004 Normand, College Station, TX 77845 (US). REDDY, Avutu, S.; 3902 E. 29th Street #G11, Bryan, TX 77802 (US). NUCCIO, Michael; P.O. Box 553, College Station, TX 77841 (US). NUNBERG, Andrew, N.; 2804 B. Sprucewood Street, Bryan, TX 77801 (US). FREYSSINET, Georges, L.; 21, rue de Nervieux, F-69450 Saint-Cyr-au-Mont-d'Or (FR). (74) Agent: MITSCHERLICH & PARTNER; Sonnenstrasse 33, D-80331 München (DE).		(81) Designated States: AU, BR, CA, CN, JP, RO, RU, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 12 September 1996 (12.09.96)
(54) Title: PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE (57) Abstract Linoleic acid is converted into γ -linolenic acid by the enzyme $\Delta 6$ -desaturase. The present invention is directed to isolated nucleic acids comprising the $\Delta 6$ -desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the $\Delta 6$ -desaturase gene. The present invention provides recombinant constructions comprising the $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

INTERNATIONAL SEARCH REPORT

Int'l Application No

PLI/IB 95/01167

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/53 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>KADER, J.-C. AND P. MAZLIAK (ED.). PLANT LIPID METABOLISM; 11TH INTERNATIONAL MEETING ON PLANT LIPIDS, PARIS, FRANCE, JUNE 26-JULY 1, 1994. XX+588P. KLUWER ACADEMIC PUBLISHERS: DORDRECHT, NETHERLANDS;; NORWELL, MASSACHUSETTS, USA. 0 (0). 1995. 509-511. ISBN: 0-7923-3250-4, XP000569979</p> <p>GALLE A-M, ET AL.: "Solubilization of DELTA-12- and DELTA-6-desaturases from seeds of borage microsomes."</p> <p>see the whole document</p> <p>---</p> <p>-/--</p>	1-28



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

4 July 1996

Date of mailing of the international search report

23.07.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+ 31-70) 340-3016

Authorized officer

Maddox, A

INTERNATIONAL SEARCH REPORT

 Int'l Patent Application No
 PCT/IB 95/01167

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>KADER, J.-C. AND P. MAZLIAK (ED.). PLANT LIPID METABOLISM; 11TH INTERNATIONAL MEETING ON PLANT LIPIDS, PARIS, FRANCE, JUNE 26-JULY 1, 1994. XX+588P. KLUWER ACADEMIC PUBLISHERS: DORDRECHT, NETHERLANDS; NORWELL, MASSACHUSETTS, USA. 0 (0). 1995. 21-23. ISBN:0-7923-3250-4, XP000569981</p> <p>SCHMIDT H, ET AL.: "PCR-based cloning of membrane-bound desaturases" see the whole document</p> <p>---</p>	1-3
Y	<p>WO,A,93 06712 (RHONE POULENC AGROCHIMIE) 15 April 1993 see the whole document</p> <p>---</p>	4-28
A	<p>BIOCHEM J 252 (3). 1988. 641-648. , XP000568812</p> <p>GRIFFITHS G: "DELTA-6 AND DELTA-12 DESATURASE ACTIVITIES AND PHOSPHATIDIC ACID FORMATION IN MICROSOMAL PREPARATIONS FROM THE DEVELOPING COTYLEDONS OF COMMON BORAGE BORAGO-OFFICINALIS." see the whole document</p> <p>---</p>	1-3
A	<p>BIOCHIM BIOPHYS ACTA 1158 (1). 1993. 52-58., XP002007452</p> <p>GALLE A M, ET AL.: "BIOSYNTHESIS OF GAMMA LINOLENIC ACID IN DEVELOPING SEEDS OF BORAGE BORAGO-OFFICINALIS L. " see the whole document</p> <p>---</p>	1-3
A	<p>PLANT MOLECULAR BIOLOGY, vol. 26, 1994, pages 631-642, XP002000999</p> <p>SCHMIDT, H., ET AL.: "Purification and PCR-based cDNA cloning of a plastidial n-6 desaturase" see the whole document</p> <p>---</p>	1-3
A	<p>JOURNAL OF THE AMERICAN OIL CHEMISTS SOCIETY, vol. 67, no. 4, April 1990, pages 217-225, XP002001000</p> <p>BAFOR, M., ET AL.: "Properties of the glycerol acylating enzymes in microsomal preparations from the developing seeds of safflower (Carthamus tinctorius) and turnip rape (Brassica campestris) and their ability to assemble cocoa-butter type fats" see page 224, right-hand column, paragraph 2</p> <p>---</p>	19-24
	<p>---</p> <p>-/--</p>	

INTERNATIONAL SEARCH REPORT

International Application No

PLT/IB 95/01167

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO,A,94 18337 (MONSANTO CO ;UNIV MICHIGAN (US); GIBSON SUSAN IRMA (US); KISHORE G) 18 August 1994 see page 32 - page 35; claim 15 ---</p>	26-28
A	<p>NATURE, vol. 347, 13 September 1990, pages 200-203, XP002001001 WADA, H., ET AL.: "Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation" see the whole document ---</p>	26-28
A	<p>PLANT PHYSIOLOGY, vol. 105, no. 2, June 1994, pages 601-605, XP002001002 KODAMA, H., ET AL.: "Genetic enhancement of cold tolerance by expression of a gene for chloroplast omega-3 fatty acid desaturase in transgenic tobacco" see the whole document -----</p>	26-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PLI/IB 95/01167

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9306712	15-04-93	AU-B- 667848	18-04-96
		AU-B- 2881292	03-05-93
		BG-A- 98695	31-05-95
		BR-A- 9206613	11-04-95
		CA-A- 2120629	15-04-93
		CZ-A- 9400817	13-09-95
		EP-A- 0666918	16-08-95
		HU-A- 69781	28-09-95
		JP-T- 7503605	20-04-95
		NZ-A- 244685	27-06-94
		ZA-A- 9207777	21-04-93
WO-A-9418337	18-08-94	EP-A- 0684998	06-12-95

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

This Page Blank (uspto)